TRIM22 IS A NOVEL RESTRICTION FACTOR OF HERPESVIRUSES

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Abstract

The host response to the family of nuclear replicating DNA viruses or the herpesviruses includes the intrinsic, innate and adaptive arms of the immune system. Intrinsic resistance is a constitutively active line of defense against virus infections and members of the Tripartite Motif (TRIM) superfamily of proteins; such as TRIM5 and TRIM19/PML in nuclear domain 10 (ND10) bodies are important restriction factors in this system. Nuclear intrinsic restriction against the prototypical DNA virus, herpes simplex virus 1 (HSV-1) includes interferon-inducible protein 16 (IFI16) and ND10 bodies. However, the viral E3 ubiquitin ligase, ICP0, encoded by wild-type HSV-1, targets these intrinsic immune proteins for degradation.

Previous reports on the anti-viral function of TRIM22, the human paralog of the prototypical TRIM5α protein, emphasized its role as a gene of the innate immune system, particularly its expression as a Type I and Type II interferon-stimulated gene and its antiviral function against retroviruses. This study shows that TRIM22 has an additional intrinsic immune role against DNA viruses, using the herpesviruses as an example of a family of DNA viruses.

We report that TRIM22 is a novel restriction factor of HSV-1 and limits HSV-1 ICP0-null virus replication. The TRIM22-mediated restriction of HSV-1

occurs after nuclear entry but prior to viral immediate-early gene transcription, by promoting histone occupancy and heterochromatinization to reduce immediate-early viral gene expression. The ICP0-rescued virus evades the TRIM22-specific restriction by a mechanism independent of TRIM22 degradation. We also demonstrate that TRIM22 inhibits other DNA viruses, including representative members of the β- and γ- herpesviruses. These results collectively show that TRIM22 acts in the nucleus, and provide evidence that TRIM22 restricts HSV gene expression by promoting histone occupancy on the viral genes.

Furthermore, we identified seven haplotypic variants of TRIM22 and propose that amino acid substitutions in the linker L2 domain and the coiled-coil domain of TRIM22 alter the magnitude of its restriction against the herpesviruses. Together, these results argue for the importance of the *TRIM22* gene as a restriction factor against herpesviruses and offer a novel avenue for further investigation on the role of *TRIM* genes in host genetic variation in herpesviral susceptibility.

Table of Contents

Dedicated to Dr. Norman Letvin,

Friend, Advisor and Leader who taught me science is all about taking risks

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Chapter 1:

INTRODUCTION TO THE INNATE IMMUNE RESPONSE TO HERPESVIRUSES

It is almost certain that by the time humans reach adulthood, they have been exposed to a herpesvirus. The herpesviruses are a large family of viruses with linear dsDNA genomes that replicate in the nucleus of host cells. They are found throughout the metazoan kingdom in diverse hosts ranging from oysters to humans [1]. This successful co-speciation can partially be attributed to the viral gene products encoded by the herpesviruses. This helps the herpesviruses to co-opt the host anti-viral immune response during primary infection and establish latency in their natural hosts. The ubiquitous nature of these viruses combined with their ability for life-long persistence in hosts makes them a rich source of information on virus-host interactions. Other biological characteristics common to herpesviruses are their arsenal of enzymes important for nucleotide metabolism, DNA synthesis and protein processing, and the long-term lysis of infected cells upon the production of infectious progeny.

1.1: *Herpesviridae* family and epidemiology

The *Herpesviridae* family is divided into the *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* sub-families. Among the approximately 130 herpesviruses identified in the environment, 9 of them are human pathogens. Herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), varicella-zoster virus (VZV) are α-herpesviruses. Human herpesvirus type-6A, -6B, -7 (HHV-6A, HHV-6B, HHV-7) and human cytomegalovirus (HCMV) are β-herpesviruses. Epstein-Barr virus (EBV) and human herpesvirus-8 (HHV-8) are γ-herpesviruses [2].

The α-herpesviruses, such as HSV-1, demonstrate a wide range of cellular tropism and their lytic replication cycle is well characterized in *in vitro* models. These viruses establish latency in the sensory ganglia of neurons. In humans, the two prototypical herpesviruses, HSV-1 and HSV-2, are known to cause diseases ranging from orofacial infections, to herpes labialis, to life-threatening encephalitis [1]. As of 2002, an estimated 90% of the worldwide population has either one or both of these viruses [3]. The prevalence of HSV-1 in adults is 65% in the United States [4] and 15.5% of non-Hispanic adults in the United States are seropositive for HSV-2 [5]. VZV is the causative agent of chicken pox during primary infection and causes herpes zoster or shingles upon reactivation from latency.

The β-herpesviruses have a more restricted range of cell tropism relative to the α-herpesviruses, and replicate at a significantly slower rate *in vitro*. These viruses establish latency in cells of the myeloid lineage. HCMV causes disease in immunocompromised individuals and transplant recipients. HCMV can also cause severe congenital disease. In the United States, 1 in 750 infants are born with or develop permanent problems due to congenital CMV infection [6].

The γ-herpesviruses have a restricted cell tropism and tend to establish latent, rather than lytic, infection in lymphoid cells. Consequently, the study of lytic replication cycle for these viruses is technically challenging. As an example of a γ-herpesvirus infection, EBV infects epithelial cells and resting B cells and is the causative agent of infectious mononucleosis. EBV has also been associated with various cancers including Burkitt's lymphomas, a subset of Hodgkin's

lymphomas, and nasopharyngeal carcinomas. Over 95% of adults worldwide have been exposed to EBV [7] and remain lifelong carriers of the virus. In addition, the geographic distribution of EBV-associated cancers offers evidence for host susceptibility to herpesviral disease. For example, the EBV-associated nasopharyngeal carcinoma shows high incidence in South-East Asia, northern Africa, Alaska and Greenland [8,9]. In addition, the incidence of EBV-positive Burkitt's lymphomas is high in equatorial Africa, Papua New Guinea and areas of Brazil [10]. However, this ethnographic distribution of EBV-associated cancers may be due to additional confounding correlative factors [11].

In conclusion, a significant proportion of the population worldwide is at risk for diseases associated with the herpesviruses due to their high prevalence and the ability to remain latent or "hidden" from the host immune response.

1.2: Herpesvirus replication cycles

The herpesvirus virion is able to replicate and target the host immune response due to the following features: (i) the innermost linear dsDNA genome devoid of histones is surrounded by (ii) the viral icosahedral capsid composed of protein subunits enclosed by (iii) an unstructured, proteinaceous layer called the tegument and (iv) an envelope containing viral glycoproteins.

1.2.1: HSV-1 lytic replication cycle

The viral lytic replication cycle proceeds as follows: (i) Attachment or binding to the target cell (ii) Entry into the cell (iii) Repression and de-repression of viral

DNA (iv) Gene expression (v) DNA replication and (vi) Assembly and egress (reviewed in [12]).

Attachment or binding to the target cell: The virion glycoproteins, gC and gD, bind to glycosaminoglycans on the target cell surface. This allows for the viral gD to interact with one of the natural receptors of HSV- nectins, herpes virus entry molecule (HVEM), and 3-O-sulfated heparan sulfate. The resulting conformational changes in gH/gL, followed by the activation of gB allows for the fusion of the viral envelope with the cell surface membrane.

Entry into the cell: After the envelope fuses with the cell membrane, the viral tegument and the enclosed capsid core transits through the cytoplasm along microtubules to the nuclear pores. The capsids dock at the nuclear pores and the viral DNA is then released into the nucleus in cooperation with host cell factors including host nuclear factor importin-β and nuclear pore complex proteins. Although some of the viral tegument proteins have different intracellular destinations, the viral VP16 tegument protein enters the nucleus.

Repression and de-repression of viral DNA: After nuclear entry, the viral DNA circularizes in a process mediated by the cellular DNA ligase IV/XRCC4. The host cell initially silences the viral DNA by assembling the free viral DNA into nucleosomes and initiating posttranslational modifications of histone tails to compact the chromatin to form heterochromatin. At later times during infection, the amount of chromatin associated with viral DNA is reduced and posttranslational modifications that reduce the compaction of viral DNA or euchromatin marks are increased. The viral tegument protein, VP16, de-represses the chromatinized viral

gene promoters with the aid of other cellular factors such as Oct-1, host cell factor 1 (HCF-1), lysine-specific demethylase 1 (LSD1), a histone acetyl transferase, CLOCK (reviewed in [12]).

Gene expression: The viral genes are transcribed in three waves, starting with the immediate-early or α genes, followed by the early or β genes and ending with the late or γ genes. The viral gene transcripts are exported out of the nucleus into the cytoplasm, where the host cell translation apparatus is hijacked to synthesize the viral proteins.

The immediate-early genes are transcribed in the absence of *de novo* protein synthesis and include infected cell polypeptide (ICP) 0, 4, 22, 27 and 47. The expression of early and late genes requires ICP4. ICP0 is particularly important as it promotes the replication of HSV-1 in experimental systems at low multiplicities of infection, as evidenced by a 10- to 1000- fold defect in viral yields of ICP0-deleted mutant viruses [13,14]. At high multiplicities of infection, this defect in viral yields is not observed in transformed cell lines. The U2OS osteosarcoma cell line is the most permissive cell line for ICP0-deleted mutant viruses and therefore, is the standard cell line for readouts of the number of infectious viral particles comparing wild-type and ICP0-mutant viruses. ICP0 transactivates both viral and non-viral gene promoters [15]. This is partially mediated by ICP0's ability to derepress viral genomes and counter the effect of host histone deacetylases (HDACs). Furthermore, ICP0 is crucial to the switch between latent and lytic replication of HSV-1 [16].

DNA replication: The early gene products are components of the viral DNA replication machinery and productive infection; the early proteins such as ICP8 reduce the expression of the immediate-early genes. The HSV-1 gene products UL9 (origin-binding protein), ICP8 (ssdNA binding protein), UL30/UL42 (viral DNA polymerase), and UL5/UL8/UL52 (helicase/primase complex) are all involved in viral DNA synthesis. Concurrent with viral DNA replication, the late gene products are transcribed and serve in the structural assembly of newly formed capsids.

Assembly and egress: The nucleocapsids transit through the nuclear membranes by multiple hypothesized processes including primary envelopment at the inner nuclear membrane, followed by deenvelopment at the outer nuclear membrane. The resultant capsids are associated with tegument proteins, followed by envelopment in cytoplasmic organelles such as the trans-Golgi network. The enveloped virus is then exocytosed out of the cell.

1.2.2: HSV-1 latent replication cycle

The HSV-1 latent replication cycle is not as well studied as the lytic replication cycle. Following the spread of HSV-1 at the primary site of infection, the virus infects sensory neurons. The nucleocapsid travels via the axonal termini to the nucleus where the viral DNA is maintained as extrachromasomal circular episomes, associated with nucleosomes. Instead of the lytic genes usually expressed in productive infection, the latency-associated transcripts (LATs) are produced. The virus undergoes reactivation or a return to lytic infection under many

conditions including stress and UV irradiation to the host. This reactivation can lead to a visual asymptomatic lesion.

1.2.3: EBV lytic replication

The lytic replication cycle of EBV is mostly informed by studies on reactivation from latency. Viral entry in epithelial cells occurs at the cell surface rather than through endocytosis. In addition to CD21, the EBV glycoprotein BMRF2 also mediates attachment to tonsilar epithelial cells. The viral immediate-early gene products, BZLF1 and BRLF1, are expressed in lytic infection. These proteins then induce the expression of the early gene products, BALF5 or the EBV DNA polymerase, BMRF1, and the ssDNA binding protein BALF2 (reviewed in [17]). The EBV genome then replicates in a rolling circle mechanism similar to HSV-1 DNA replication in discrete nuclear compartments. This is followed by late gene expression resulting in the production of viral capsid antigen, major capsid protein and glycoproteins. Linear individual units of viral genomes are assembled with the structural capsid proteins and the newly assembled nucleocapsids transit through the cytoplasm and exit the cells.

1.2.4: EBV latent replication cycle

Unlike HSV-1, EBV primarily undergoes the latent replication cycle in *in vitro* systems of infection. The ability of the virus to establish latency is closely tied with the number of EBV-associated cancers mentioned earlier.

Attachment: In B cells, the EBV virion glycoproteins gp350/220 bind to CD21, a complement C3d receptor, to tether the virus onto B cells. Further binding in B cells is mediated by the interaction between the viral gp42 and the cellular HLA-II (Including HLA-DP, HLA-DQ and HLA-DR) [18,19]. This is followed by fusion mediated by viral gH/gL and endocytosis of the attached virion.

Transformation of B cells: After infection of B cells, the host RNA polymerase II transcribes viral genes. Early in viral replication, the Wp promoter drives the expression of the EBV gene products, Epstein Barr nuclear antigen-2 (EBNA-2) and Epstein Barr nuclear antigen leader protein (EBNA-LP). The virus establishes different patterns of gene expression in stages designated as latency 0, latency 1, and latency 2. The major viral gene products expressed during this stage are EBV encoded RNAs (EBERs), microRNAs, Epstein Barr nuclear antigens (EBNAs) and latent membrane proteins (LMPs) found in cellular membranes.

In latency 0, or the stage in which the virus is found in B-lymphocytes of infected patients, very few viral gene products are expressed. In latency 1 found in Burkitt's lymphoma, only EBNA1 is expressed. In latency 2 found in nasopharyngeal carcinomas, Hodgkin's lymphomas, T-cell lymphomas, EBNA1, LMP1 and LMP2 are expressed. In latency 3, the virus establishes latency in B cells, which get immortalized and proliferate as lymphoblastoid cell lines (B-LCLs). All the known latent genes are expressed in B-LCLs including EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, LMP1, LMP2 and BARTs and EBV encoded RNAs such as EBER-1 and EBER-2. EBNA-LP and EBNA-2 reach stable levels at 24-32 hours post infection (hpi), which are maintained in B-LCLs (reviewed in [12]).

Episome formation and maintenance: The viral genomes are maintained in B-LCLs as multicopy, extrachromosomal, covalently closed circular DNA genomes called episomes in the nucleus during latency. These episomes are associated with chromatin in the form of nucleosomes and are maintained in stable copy numbers and passed to daughter cells through non-stochastic segregation by an EBNA1-mediated mechanism [20-23]. Besides EBNA1, proteins involved in the DNA replication fork protection, Timeless (Tim) and Timeless-interacting protein (Tipin) are essential for the maintenance of episomal forms of EBV DNA in latent cells [24].

1.3: Immune response to herpesviruses

The immune response to the herpesviruses consists of the adaptive, innate, and intrinsic arms. The adaptive immune responses include cell-mediated immunity and antibody production. The innate immune response includes the induction of complement, activation of natural killer (NK) cells and macrophages, production of interferons (IFNs) by plasmacytoid dendritic cells (pDCs), conventional dendritic cells and macrophages. Prior to the induction of these various responses, the host response includes intracellular innate immune recognition of the herpesviruses. The innate immune sensing of HSV-1 is described below as a relevant example.

1.3.1: Innate immune response to herpesviruses

The patterns of the virus that are recognized as foreign, referred to as pathogen-associated molecular patterns (PAMPS) include proteins such as viral

glycoproteins and tegument; nucleic acids such as the GC-rich DNA genome and RNA [25]. The host cell has pattern recognition receptors (PRRs), which recognize these PAMPs, as part of the innate immune response. Examples of PRRs include members of the Toll-like receptor (TLR) family, NOD-like receptors (NLRs) and RIG-I like receptors (RLRs). This results in signaling mediated by adaptor proteins, the activation of transcription factors such as interferon regulatory factor (IRF) -3, -7, Activator Protein 1 (AP1) and nuclear factor κB (NF-κB). Consequently, this results in the induction of Type I IFNs and other cytokines [26].

Upon viral adhesion to the host cell, viral glycoproteins gH/gL and gB are recognized by Toll-like receptor 2 (TLR2), resulting in NF-κB activation and the production of pro-inflammatory cytokines in 293T cells [27]. Endosomal TLR3 recognizes dsRNA and endosomal TLR9 recognizes unmethylated CpG DNA. This triggers the production of IFNβ, IFNλ and IL6.

The viral DNA transitions through the cytoplasm where it is recognized by cyclic GMP-AMP synthase (cGAS) [28], DEAD (Asp-Glu-Ala-Asp) Box Polypeptide 60 (DDX60), DEAH (Asp-Glu-Ala-His) Box Helicase 9 (DHX9) [29], DHX36 [29] or Ku70, which leads to the production of cytokines such as Type I IFN. Viral DNA is also recognized in endosomes by TLR9 upon endocytosis in plasmacytoid dendritic cells. Upon nuclear entry, viral DNA is recognized by IFI16 resulting in the IRF3 mediated production of IFNβ [30] and caspase activation through a hypothesized inflammasome-mediated pathway [31].

The prototypic member of the RIG-I-like receptors (RLRs) RIG-I, detects the 5'-triphosphate RNA transcribed from the HSV-1 DNA by RNA pol III. Melanoma differentiated gene 5 (MDA5), another RLR family member, recognizes higher order RNA intermediates in the cytoplasm transcribed from viral DNA resulting in IFNβ, IFNλ and TNFα production [32]. Therefore, it is evident that the host cell mounts a significant immune response against infection by DNA viruses.

The importance of the intracellular innate immune response to the herpesvirus is evidenced by the fact that susceptibility to herpesviral diseases increases in hosts with mutations in genes of this pathway. Mutations in components of the TLR3 innate immune signaling pathway in young children aged 3 months – 6 years old increase the risk of recurrent herpesvirus encephalitis. These mutations in *TLR3* [33], *TRAF3* [34], *UNC93B1* [35] and *TRIF* [36] genes are hypothesized to reduce the capacity of these patients to produce Type I IFNs. Interestingly, these individuals do not seem to show an increased susceptibility to other pathogens suggesting that the deficiency in the TLR3 signaling pathways is specific to herpesviral susceptibility in these patients [37].

The importance of the extracellular innate immune response is evident in rodent models of herpesviral infections. Resistance to MCMV, a murine β-herpesvirus, was attributed to the *Cmv4* gene locus encoding the NK cell receptor, Ly49H [38]. The expression of Ly49H on NK cells of C57Bl/6 mice also contributes to the resistance of these mice to MCMV infection, whereas the

absence of the Ly49H receptor on Balb/c mice contributes to the susceptibility of these mice to MCMV.

Despite the significant host immune response to herpesviruses, these viruses have a noteworthy amount of genomic space to encode viral proteins that help evade the immune response. For example, HSV-1 counters the immune response in the following ways: (i) inhibition of host transcription (ii) degradation of host messenger RNAs (mRNAs) and the resultant depletion of short-lived cellular proteins by the virion host shutoff (vhs) protein (iii) overcoming the cell-mediated repression of viral DNA (iv) degradation of cellular proteins needed for the immune response by the infected cell polypeptide 0 (ICP0) (v) disruption of host mRNA splicing (vi) prevention of apoptosis and autophagy (vii) inhibition of antigenic presentation (viii) inhibition of cellular cytotoxicity implemented by the immune system (reviewed in [12]).

1.3.2: Intrinsic response to the herpesviruses

Unlike the intracellular innate response, which requires signaling upon viral recognition, the cell's intrinsic resistance system is setup for an immediate response to viral infection. It consists of germline encoded, constitutively expressed factors which do not require additional signaling to respond to viral infection [39]. In addition to this intrinsic role, a number of these components are also induced by IFN. The components of the intrinsic immune system have been well established in studies of *in vitro* tropism for retroviruses- primarily the identification of Friend virus susceptibility gene (*Fv1*) in the restriction of the

murine leukemia virus (MLV). Other examples of the intrinsic immune system include the APOBEC family of RNA-editing cytidine deaminases. These enzymes convert viral cytidine to uridine. Another example is tetherin, which has been shown to prevent HIV-1 viral budding, and members of the TRIM superfamily of proteins, which differ in their mechanisms of viral inhibition.

In the context of herpesviruses, the intrinsic immune response consists of nuclear domain 10 (ND10) bodies [40]. In addition to being upregulated by IFN, these are well-studied intranuclear organelles that confer resistance to the herpesviruses. They consist of cellular proteins including TRIM19/ promyelocytic leukemia protein (PML), Sp100, hDaxx and α-thalassemia/mental retardation syndrome X-linked protein (ATRX) [40]. hDaxx has been shown to repress human cytomegalovirus (HCMV) and a replication-defective version of HCMV [41]. Both hDaxx and ATRX have been shown to be important in chromatin modification and viral gene repression, particularly in HSV-1 and HCMV infection [42]. PML will be discussed in further detail later.

Another intrinsic mechanism of herpesviral inhibition is the silencing of viral DNA upon nuclear entry by IFI16. The depletion of IFI16 has been shown to promote the replication of an ICP0-null virus [43]. This was due to a reduction in the heterochromatin marks and an increase in euchromatin marks associated with the viral gene promoters [43]. Much like PML, IFI16 is also upregulated by Type I IFN.

Furthermore, cellular factors involved in the DNA damage repair response are also capable of recognizing viral genetic material [44] and localize to sites of

incoming viral genomes independently of ND10 components [45]. Although components of the Ataxia telangiectasia mutated (ATM)-dependent signaling pathway such as ATM and Mre11 are beneficial for lytic replication [46], the ubiquitin ligases RNF8 and RNF168 are recruited to incoming viral genomes [47]. These proteins in turn recruit 53BP1, a known DNA damage repair protein, in an attempt to restore what the cell perceives as “damaged” DNA. Although the effect of these proteins on viral replication is yet to be elucidated clearly, these DNA damage repair proteins provide a repressive environment for incoming genomes [45]. The absence of Ku70, a protein involved in non-homologous end joining, in mouse embryonic fibroblasts increases HSV-1 viral replication [48].

Much like the viral mediated escape of the innate immune response, the herpesviruses encode proteins that confer escape from the intrinsic immune response. As an example, the HSV-1 ICP0 counters the host immune response by disruption of ND10 bodies, degradation of the components of ND10 bodies such as PML and Sp100 [49] and degradation of IFI16 [30]. ICP0 has also been shown to counter the DNA damage response by targeting the degradation of the DNA repair proteins, RNF8 and RNF168 [47]. Furthermore, ICP0 has been demonstrated to sequester the accumulation of a transcription factor important for the induction of Type I IFN in the nucleus, or IRF3 [50]. These functions of ICP0 are partially attributed to its N-terminal RING domain, which contains E3 ubiquitin ligase activity.

1.3.3: TRIM proteins

Members of the Tripartite Motif (TRIM) superfamily of proteins are known mediators of the intrinsic immune response (reviewed in [51]). They are found throughout the metazoan kingdom with over 60 proteins identified in humans. Functionally, they are involved in multiple cellular processes including apoptosis, cell differentiation and proliferation, with a number of them involved in anti-viral activity. The order and spacing across all TRIM proteins are conserved, in that they share a common structure of a RING ('really interesting new gene') domain followed by one or two B-box domains, a coiled-coil domain and a variable C-terminal domain.

The RING domain consists of 40-60 amino acid residues and is capable of binding zinc atoms [52]. The RING domain is hypothesized to have E3 ubiquitin ligase activity in all TRIM proteins, and this has been validated in TRIM5δ and TRIM5α isoforms, TRIM18/MID1, TRIM22, TRIM25, TRIM32 and TRIM35 (reviewed in [53]). In addition, the RING domain of TRIM19/PML has been shown to associate with the SUMO-conjugating enzyme, UBE21, demonstrating its potential role in SUMOylation (reviewed in [54]).

The TRIM proteins also have one or two B-box domains, consisting of two CHC_3H_2 zinc-finger motifs consisting of 40 residues each (reviewed in [53]). They have been shown to confer anti-viral specificity and higher-order oligomerization in the context of TRIM5 to promote capsid-binding [55],[56]. However, a consistent role for the B-box domains across the different TRIM proteins is yet to be elucidated (reviewed in [53]).

The coiled-coil domain is a secondary protein structure comprised of α-helices and promotes homo-oligomerization (reviewed in [53]). The presence of the coiled-coil domain has been shown to be important for TRIM5's ability to trimerize and function as a restriction factor [57].

Although the C-terminal domain varies across the different TRIM proteins, 40% of the 66 TRIM proteins identified have a B30.2/SPRY domain (related to the immunoglobulin folds of the butyrophilin protein) ([58], reviewed in [53]). Therefore, the domains of the TRIM proteins have been shown to clearly impact their anti-viral function. The following sections address the anti-herpesviral role of specific TRIM proteins.

1.3.3.1: TRIM19/ PML

The initial characterization of TRIM19 was due to a chromosomal translocation in acute promyelocytic leukemia patients, resulting in a fusion protein with retinoic acid receptor-α (RAR-α) [59] [60,61]. The promyelocytic leukemia protein (PML) is involved in a variety of cellular processes including apoptosis, cell proliferation, transcriptional and translational regulation, signal transduction and the DNA damage response (reviewed in [53], [62]). It is hypothesized that the variation in the C-terminal domain of the splice variants confers the ability of PML to interact with a variety of different proteins (reviewed in [53]). PML is important for the integrity of nuclear domain 10 (ND10) bodies in the nucleoplasm [63], in which it is associated with a number of other proteins including hDaxx/ATRX and Sp100.

PML has both innate and intrinsic functions: It has been shown to regulate Type II IFN (IFNγ) signaling [64], and is also upregulated by Type I IFN resulting in an increase in the number and size of ND10 bodies. It confers resistance to RNA viruses such as HIV-1, vesicular stomatitis virus [65,66] and influenza A virus. PML has been shown to inhibit the replication of a number of DNA viruses including parvoviruses [67], HCMV and an ICP0-null mutant of HSV-1.

1.3.3.2: TRIM5

TRIM5 is a well-studied TRIM protein that has been shown to restrict HIV-1 in Old World monkeys. HIV-1 restriction was previously shown to be a function of cross-species restriction factors, Ref 1 and Lv1 ([68-72], reviewed in [67]). The TRIM5α isoform in rhesus macaques (rhTRIM5α) has been shown to restrict HIV-1 by capsid recognition and accelerated uncoating of the capsid and has been shown to be a novel PRR for viral capsid [73,74]. TRIM5 in humans has some anti-viral activity against HIV-1. However, this does not show the same level of potency as rhTRIM5α [75-77].

The different domains of TRIM5α have been shown to modulate anti-viral activity in the context of HIV-1 restriction [55,57,78-82]. It was also found that a single point mutation in the C-terminal SPRY domain of human TRIM5 could confer restriction against HIV-1[77]. In addition, expression levels of the human TRIM5 and polymorphisms in the *TRIM5* gene can also confer resistance against HIV-1 [83,84]. Therefore, the literature describing anti-retroviral role of TRIM5 is well established.

However, the role of TRIM5 in DNA viruses such as herpesviruses was only recently elucidated. Rhesus fibroblasts show a 2-log defect in HSV-1 KOS replication and a 3-log defect in HSV-2 replication relative to the human cell line, HeLa [85]. Upon further investigation, Old World monkey TRIM5α, particularly rhTRIM5α, inhibited HSV-1 replication by a few-fold at low multiplicities of infection (MOIs), and this is overcome at higher MOIs. This MOI-dependent effect on viral restriction is consistent with previous reports of the TRIM5-mediated restriction of HIV-1 [74]. The TRIM5-mediated effect was at an early stage in the viral life cycle prior to immediate-early gene expression. Furthermore, the Letvin laboratory demonstrated that haplotypic variation in *TRIM5* altered the levels of herpesviruses in rhesus macaque cells; rh*TRIM5* genes encoding a two amino acid deletion in the B30.2/SPRY domain demonstrated lower permissivity to β- and γ- herpesviruses, particularly rhesus CMV and rhesus LCV, than rhesus macaque B-LCLs encoding the *TRIM5* with wild-type B30.2 domain [86]. These studies established a premise for the study of *TRIM* genes relative to herpesvirus susceptibility.

1.3.3.3: TRIM22

TRIM22 or Stimulated Trans-acting factor of 50kDa (Staf50) located on chromosome 11p15 was initially identified as a Type I and Type II interferon-stimulated gene in a human lymphoblastoid cell line (B-LCL) [87,88]. It was further hypothesized to function as a nuclear E3 ubiquitin ligase [89]. Consistent with the antiviral role of a number of interferon-stimulated genes, multiple reports

have established that TRIM22 is upregulated by IFNα [90-92], PEGylated-IFNα [93], IFNβ [92,94], IFNγ [95,96] and upon rubella virus [97] and HIV-1 infection [97,98]. In the absence of exogenous IFN, TRIM22 is expressed at high levels in immune cells including resting T cells [99]. TRIM22 is consistently repressed in activated T cells [99] and was found to be down-regulated in $CD4^+$ lymphocytes from a patient with systemic lupus erythematous (SLE), an autoimmune disorder [100].

Besides its additional role as an activator of the non-canonical NF-κB pathway [101,102], other reported roles of TRIM22 include its role as an anti-proliferative and differentiation-associated gene. It has been shown to be a p53 target gene, to inhibit clonogenic growth of U937 leukemic cells, and to have decreased expression during progression of $CD34^+$ human bone marrow progenitor cells to late erythroid maturation [103].

There is evidence for a role for TRIM22 in humans, which include the following: (i) Down-regulation of TRIM22 was shown to be associated with relapse in patients with Wilms' tumors or nephroblastomas [104] and increased mortality [105]. (ii) TRIM22 expression correlated negatively with plasma viral loads in HIV-1 infected patients [106] and correlated positively with CD4+ T cell counts in these patients [92]. (iii) TRIM22 has been demonstrated to have significant anti-viral activity against human immunodeficiency virus-1 (HIV-1) [87,92,94,107,108], encephalomyocarditis virus (ECMV) [109], hepatitis B virus (HBV) [110], hepatitis C virus (HCV) [90], and influenza A virus (IAV) [91]. Most of the antiviral function of TRIM22 has been attributed to the E3 ubiquitin ligase

activity of its pertinent RING domain [89]. It has been shown to mediate the ubiquitination of the ECMV 3C protease, which is crucial for the replication of this single-stranded RNA virus [109], to target the influenza A virus nucleoprotein for degradation [91] and to interrupt HCV replication by ubiquitination of the viral NS5A [90].

The reports on the mechanism of TRIM22-mediated restriction of HIV-1 are unclear. TRIM22 has been shown to inhibit HIV-1 LTR transcription in a RING-domain independent manner [108]. However, mutating the cysteine residues at positions 15 and 18 residues in the RING domain of TRIM22 prevented the trafficking of Gag protein to the plasma membrane, and thus abrogated the budding of HIV-1 particles [94]. It has also been shown to inhibit the HBV core promoter activity in a RING domain-mediated mechanism. However, the role of the RING domain in this study is due to the nuclear localization conferred by this domain, rather than its E3 ubiquitin ligase activity [110].

Interestingly, some viruses potentially hijack the TRIM22's anti-viral properties to prevent super infection upon establishment of latency. TRIM22 was shown to be upregulated in cell lines expressing the γ-herpesvirus, KSHV latency-associated nuclear antigen (LANA) [111] and EBV latent membrane protein 1 (LMP1) [112]. Therefore, TRIM22 as an innate immune gene and other functional properties are well established, however its role as a restriction factor has not been elucidated.

1.3.3.4: Evolutionary history of the *TRIM5* and *TRIM22* gene neighbors

The virus-host conflict can be characterized by rapid changes at the host-virus interface in anti-viral genes such as *TRIM5*. The ratio of rate of occurrence of non-synonymous amino acid substitutions (*dN*) to the rate of occurrence of synonymous amino acid substitutions (*dS*) or the *dN/dS* ratio is a means of quantifying protein evolution [113]. *dS* is fixed by random genetic drift and is neutral. Therefore, if *dN* exceeds *dS*, then the interpretation is that positive selection fixes non-synonymous amino acid substitutions faster than neutral ones. Conversely, if *dN* is lower than *dS*, then purifying selection removes a proportion of the non-synonymous amino acid substitutions and refers to high conservation.

The anti-viral *TRIM5* gene sits in a gene cluster with *TRIM6*, *TRIM34* and *TRIM22* at position 11p15.4. Evolutionary analysis of the primate *TRIM5* gene of 17 primate genomes spanning 33 million years of evolution demonstrated that it was positively selected for, predating the origin of primate lentiviruses, which have been hypothesized to originate <1 million years ago [114,115]. The authors suggested that the episodic positive selection of the *TRIM5* gene in primates is influenced by interaction with endogenous retroviruses and retrotransposition events. This positive selection was concentrated on residues in the C-terminal SPRY domain, a region known to be important for lentiviral interaction [114].

Further investigation of the *dN/dS* parameter of the *TRIM5* and *TRIM22* gene neighbors in human, rhesus macaque and chimpanzee genomes showed that *TRIM22*, much like *TRIM5*, is also positively selected for in primates [116].

Further analysis of the *TRIM22* genes in 21 primate genomes spanning 33 million years of primate divergence provided strong support for the positive selection of *TRIM22* in the hominoid and Old World monkey clades, suggesting that it has been positively selected since 23 million years ago [116].

Interestingly, the *TRIM5* and *TRIM22* gene neighbors underwent discordant evolution with either one or the other being positively selected for in the hominoid and Old World monkey species investigated [116]. In addition, the cow genome includes an expanded cluster of *TRIM5* genes but no *TRIM22* gene, while the dog genome encodes *TRIM22* but does not encode *TRIM5* [116]. These findings all suggested that *TRIM22*, much like *TRIM5*, has the evolutionary trademarks of an intrinsic immune gene.

Given the prior evidence that *TRIM5* in rhesus macaques restricts HSV-1, and haplotypic variability in the rh*TRIM5* gene associates with lower rhCMV and rhLCV viral loads, we wanted to address whether TRIM22 variability affects human susceptibility to herpesviruses. The established anti-viral properties of TRIM22 in many hosts support our hypothesis that TRIM22 may also affect viral infection in humans. Therefore, we investigated the potential restrictive ability of *TRIM22* in HSV-1, HCMV and EBV.

Chapter 2:

TRIPARTITE MOTIF (TRIM22) RESTRICTS HERPES SIMPLEX VIRUS 1 BY EPIGENETIC SILENCING OF IMMEDIATE-EARLY VIRAL GENES

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Author contributions: T.S.R., N.L.L., and D.M.K., designed research; T.S.R. and P.E.M. performed research; T.S.R. and D.M.K. analyzed data; T.S.R. and D.M.K. wrote the paper.

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Abstract

Intrinsic resistance is a crucial line of defense against virus infections and members of the Tripartite Motif (TRIM) family of proteins are major players in this system, particularly TRIM19/PML in ND10 bodies. Previous reports on the anti-viral function of another TRIM protein, TRIM22, emphasized its innate immune role as a Type I and Type II interferon-stimulated gene against RNA viruses. This study shows that TRIM22 has an additional intrinsic role against DNA viruses. Here, we report that TRIM22 is a novel restriction factor of HSV-1 and limits ICP0-null virus replication by modulating histone occupancy and heterochromatin in shutting off immediate-early (IE) viral gene expression. The corresponding wild-type equivalent of the virus evades the TRIM22-specific restriction by a mechanism independent of ICP0-mediated degradation. We also demonstrate that TRIM22 inhibits other DNA viruses, including representative members of the β- and γ- herpesviruses. Collectively, these results strengthen the case for intrinsic resistance to foreign DNA in the nucleus and argue that TRIM22 is a hitherto, unknown component of this system.

Introduction

The intrinsic immune system consists of constitutively expressed, germline encoded restriction factors that provide an immediate anti-viral response in host cells [39]. This system includes members of the Tripartite Ring Interaction Motif (TRIM) proteins, which have important roles in viral inhibition in addition to being involved in diverse cellular processes [102,103,117-119]. In the context of the nuclear-replicating DNA virus family, the herpesviruses, nuclear domain 10 (ND10) bodies are well-established sub-organelles that confer resistance to the ubiquitous herpesviruses [120]. They consist of cellular proteins including TRIM19/promyelocytic leukemia protein (PML), Sp100, hDaxx and α-thalassemia/mental retardation syndrome X-linked protein (ATRX) [121]. PML, Sp100 and hDaxx depletion increases the infectivity of an ICP0-null herpes simplex virus-1 (HSV-1) virus, and Sp100 has been implicated in the repression of HSV-1 gene expression [122]. ATRX and hDaxx depleted cells also demonstrate increased plaque formation and gene expression of an ICP0-null HSV-1 virus [42]. HDaxx has been shown to repress human cytomegalovirus (HCMV) and a replication-defective version of HCMV [123]. Both hDaxx and ATRX have been shown to be important in chromatin modification and viral gene repression, particularly in HCMV infection [124].

To counteract the restriction exerted by the various known intrinsic immune factors, the herpesviruses encode a number of proteins that allow for virus propagation, even in relatively hostile conditions implemented by the host interferon (IFN) responses [12]. This includes the infected cell polypeptide 0

(ICP0), which degrades components of the intrinsic immune system, such as γ-interferon-inducible protein 16 (IFI16) and components of the nuclear ND10 bodies [12,30,125-127]. ICP0 also sequesters interferon regulatory factor 3 (IRF3) from the *IFNB* gene promoter, thus preventing the expression of this Type I IFN [12,128,129]. In addition, ICP0 counters host chromatin silencing mechanisms, allowing for the de-repression of early (E) genes [130,131]. As a result, ICP0-null viruses demonstrate a 10 to 100-fold replication defect relative to the wild-type viruses in primary cells [132,133]. Although depletion of known restriction factors of HSV-1 such as PML and IFI16 rescue a significant proportion of the defect in ICP0-null virus replication, we hypothesized that there were other factors that could contribute to this restriction.

One such candidate was the human TRIM22 protein, which is the paralog of the prototypical TRIM protein- rhesus macaque TRIM5α (rhTRIM5α) [134]. RhTRIM5α was reported to inhibit lentiviruses, and also HSV-1 [135]. Sequence analyses of rh*TRIM5* and human *TRIM22* genes from 27 primate genomes demonstrated that both *TRIM22* and *TRIM5* underwent positive selection such that either *TRIM5* or *TRIM22* was selectively modified within a given species, arguing for selective evolutionary pressure exerted by species-specific pathogens [116]. These studies suggested that TRIM22 in humans has a similar functional anti-viral response against viruses, similar to rhTRIM5α. In fact, TRIM22 is an interferon (IFN)-inducible protein that has antiviral activity against a range of viruses, particularly RNA viruses [91,94,108,109] and a DNA virus, the hepatitis B virus (HBV) [110]. In addition, TRIM22 expression has also been

correlated with the induction of an anti-viral state upon establishment of latency in members of the γ-herpesvirus family such as Kaposi's sarcoma herpes virus (KSHV) (23) and the Epstein-Barr virus (EBV) [112,136]. Furthermore, TRIM22 was previously reported to be upregulated in the first 24h of HCMV infection in HFFs [137]. However, the role of TRIM22 as an intrinsic immune factor in HSV-1 infection has not been investigated.

Here, we report that TRIM22 limits ICP0-null virus replication by modulating histone occupancy and heterochromatin in shutting off immediate-early (IE) viral gene expression. The ICP0-rescued virus partially evades the TRIM22-specific restriction by a mechanism independent of ICP0-mediated degradation. In this study, we report that TRIM22 rescues part of the replication defect of the ICP0-null virus. In addition, we also find that the restrictive effect of TRIM22 is not limited to HSV-1 but is also seen with other viruses that replicate in the nucleus, such as HCMV and EBV. These results strengthen the case for TRIM22 as a novel component of the intrinsic immune response to the herpesviruses.

Materials and Methods

Cell Culture and Viruses. HFFs (Catalog number: CRL-1634), U2OS, HeLa and HEK293 were obtained from the American Type Culture Collection. HFFs were cultured as described previously [43]. U2OS and HeLa cells were grown in DMEM supplemented with 5% (vol/vol) heat-inactivated fetal bovine serum (FBS) and 5% (vol/vol) heat-inactivated bovine calf serum (BCS). HEK293 cells stably transduced with CD21 and HLA-II [138] were obtained from the Fingeroth laboratory and cultured as previously described in selection media. The ICP0-null (7134) and rescued 7134R viruses were grown and titrated on U2OS cells in parallel [139]. Donald Coen provided HCMV AD169 and it was propagated as described previously [140].

Virus Infections. HSV was diluted in PBS solution containing 0.1% glucose and 1% heat-inactivated BCS. Cells were infected at the stated MOI for 1h at 37°C, washed twice with PBS solution, and overlaid with DMEM containing 1% heat-inactivated BCS. Infected cells were incubated at 37°C for the indicated length of time. HCMV AD169 stocks were diluted in DMEM containing 10% FBS and cells were infected at the stated MOI for 1h at 37°C, washed twice with PBS solution, and overlaid with DMEM containing 10% FBS. Infected cells were incubated at 37°C for the indicated length of time. EBV-GFP stocks were prepared from a cell line kindly donated by Wolfgang Hammerschmidt as previously described [141], and the virus was diluted in DMEM containing 10% FBS and cells were infected for 5 days at 37°C.

siRNA Transfections. Double-stranded *TRIM22*-specific, and non-target control siRNAs were purchased from Dharmacon. The pooled siRNAs were transfected into HFFs using the DharmaFECT 2 transfection reagent (Dharmacon) at a final concentration of 50nM according to manufacturer's instructions. Cells were split into two separate wells at 72h post-transfection and transfected again as described above. Cells were infected after the second round of 72h post-transfection. The siRNA was replaced 24hpt with incubation media in both rounds of transfection, and cells were assayed for TRIM22 or 18S levels by RT-qPCR and immunoblot at 72hpt after the second round of transfection.

Plasmids and DNA Transfection. The TRIM22 cDNA was extracted from B lymphoblastoid cell lines from the Hap Map project and cloned into the pLPCX vector backbone (Clontech). The plasmids encoding the TRIM22 constructs lacking the different domains were a kind gift from Dr. Valerie Lin [142]. HeLa cells were plated and transfected with the Effectene reagent according to the manufacturer's instructions. Transfected cells were either treated with PBS or hIFNα-2a at 24 hours post transfection (hpt) and infected 24h post-treatment.

Cellular RNA Analysis by qPCR. Total RNA was extracted using the Qiagen RNeasy Kit and DNase treated using the DNA-free kit (Ambion). Equal amounts of RNAs were reverse-transcribed and quantified by real-time qPCR by using the Fast Power SYBR Green PCR master mix and Step One PCR sequence detection system (Applied Biosystems). qPCR reactions were carried out in

triplicate, and relative copy numbers were determined by comparison with standard curves. Mock reverse-transcribed samples were included as negative controls. Transcript levels were normalized to 18S rRNA levels and made relative to mock-infected samples.

Nuclear DNA Analysis. Nuclei from 7134 or 7134R-infected cells were isolated using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo-Scientific), and DNA was harvested from these nuclei by using a DNeasy blood and tissue column kit. Viral DNA levels were determined by RT-qPCR.

Western Blots. Cells were lysed in NuPAGE LDS Sample Buffer, and proteins were resolved on NuPAGE 4% to 12% Bis-Tris gels (Invitrogen). Proteins were transferred overnight to nitrocellulose membranes and blocked with 5% milk in PBS solution containing 0.1% Tween-20 (PBS-T). Membranes were probed with primary antibody at 4°C overnight, washed with PBS-T and incubated in secondary antibody for 1h at room temperature. Western blots were developed using the Super Signal Pico or Forte Chemiluminescence substrate.

Indirect Immunofluorescence. HeLa cells grown on coverslips were transfected with the TRIM22 mutants and fixed and permeabilized as previously described. Briefly, cells were fixed with 3.7% formaldehyde for 15 minutes on ice and permeabilized with 0.2% Triton-X100 for 15 minutes at room temperature [142]. Cells were blocked overnight with DMEM-02 and incubated with anti-FLAG M2

mouse (Sigma-Aldrich) for 1h at room temperature, washed twice with PBST and once with PBS. Fluor 488-conjugated secondary antibodies (Invitrogen) were incubated with cells for 1h at 25°C in dark. The coverslips were washed as described earlier and DAPI at a dilution of 1:1000 was added during the second PBST wash. Coverslips were mounted in ProLong Gold antifade reagent (Invitrogen). Images were acquired by using a confocal microscope (Olympus) with a 60x objective and in a single Z plane. Images were arranged in figures by using Adobe Photoshop CS4 (Adobe Systems).

Flow Cytometry. HEK293 cells infected with EBV-GFP were trypsinized, collected by centrifugation and resuspended in solution containing Aqua-Amine. Cells were washed twice in PBS and resuspended in fixing solution for 15 minutes at 4°C. Cells were counted using a flow cytometer and live cells were gated for using an Aqua-Amine negative gate after exclusion of doublets on forward and side scatter axes. GFP+ gate was defined on mock-infected cells. Data analysis was performed using FlowJo (version 8) software and graphs were constructed by using Graph Pad Prism software.

Chromatin Immunoprecipitation. HFFs were transfected with siRNAs as described earlier and after the first round of transfection, $5.5x10^5$ cells were plated in 60mm dishes and transfected for the second round of transfection. Cells were infected at 72hpt, and the ChIP protocol was conducted as previously described [143]. Prior to immunoprecipitation, 10ul of the sample is reverse

cross-linked as previously described and DNA is extracted and gel electrophoresis is conducted on a 1% gel to ensure DNA fragments are ~500bp in length. Immunocomplexes were immunoprecipitated overnight at 4°C with 2.5μg of anti-histone H3 IgG (Abcam) and anti-histone H3K9me3 (Active Motif).

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Results

Depletion of TRIM22 rescues ICP0-null HSV replication in normal human foreskin fibroblasts

To determine the role of TRIM22 as an interferon-stimulated gene (ISG) in HSV-1 replication, we measured virus yields of the HSV-1 7134 ICP0-null virus or the corresponding 7134R rescued virus in TRIM22-depleted fibroblasts under conditions of Type I IFN pre-treatment. We transfected primary human foreskin fibroblasts (HFFs) with pooled siRNAs specific for TRIM22 or non-targeting siRNAs twice to deplete *TRIM22* transcripts. To upregulate TRIM22, we treated the transfected cells with either IFNα (1000U/ml) or a PBS control 24h prior to infection with the 7134 or 7134R viruses (MOI=5) and measured virus yields at 24hpi. Upon comparison of TRIM22 transcripts in control-depleted and TRIM22-depleted fibroblasts, the knockdown efficiency was approximately 84% in the PBS pre-treated cells and 87% in the IFNα pre-treated cells (Figure 2.1A). The levels of TRIM22 protein were not detectable by western blotting in TRIM22-depleted HFFs, even in the IFNα pre-treated HFFs (Figure 2.1B). We observed an increase in 7134 virus yields in TRIM22-depleted HFFs relative to control-depleted HFFs under conditions of PBS pre-treatment (Figure 2.1C). IFNα pre-treatment reduced 7134 virus yields by approximately 10-fold relative to PBS pre-treatment in HFFs, and TRIM22 depletion did not rescue IFNα-mediated inhibition of 7134 (Figure 2.1C). The magnitude of the TRIM22-mediated inhibition on the 7134 virus was possibly due to a synergistic effect of all the individual TRIM22-specific siRNAs used (Figure 2.2A). Interestingly, at a low MOI

(0.1 pfu/cell), TRIM22 depletion rescued a substantial portion of the IFNα- and IFNγ-mediated inhibition of the 7134 virus replication (Figure 2.3). These results argued that TRIM22 inhibits the replication of the ICP0-null virus and mediates some of the anti-viral effect of IFNα at a low MOI.

Figure 2.1: TRIM22 depletion increases HSV-1 viral yields. HFFs were transfected with siRNA pools specific for TRIM22 or non-targeting siRNAs. Transfected HFFs were treated with PBS or hIFNα-2a at 1000U/ml for 24h and then were infected with HSV-1 ICP0-null (7134) or a rescued virus (7134R) at an MOI of 5. TRIM22 transcript levels were measured by RT-qPCR with primers specific for TRIM22 and 18S (n=3) (A) and protein levels were determined by immunoblotting for TRIM22 and GAPDH (B) at time of infection. Virus yields were measured 24hpi by plaque assays on U2OS cells (n=3) (C). Error bars represent standard errors of the means (*P<0.05, Student's t-test).

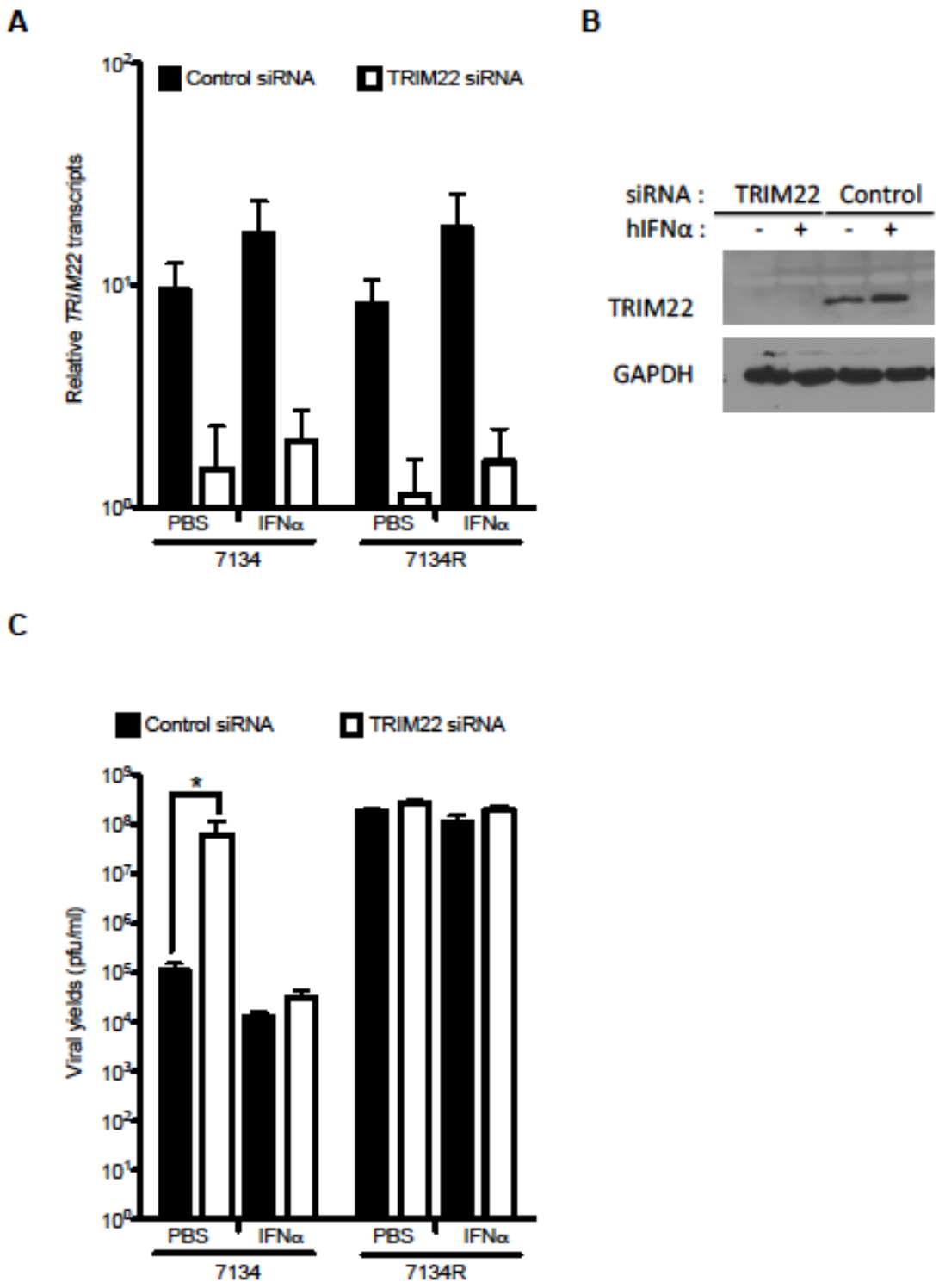
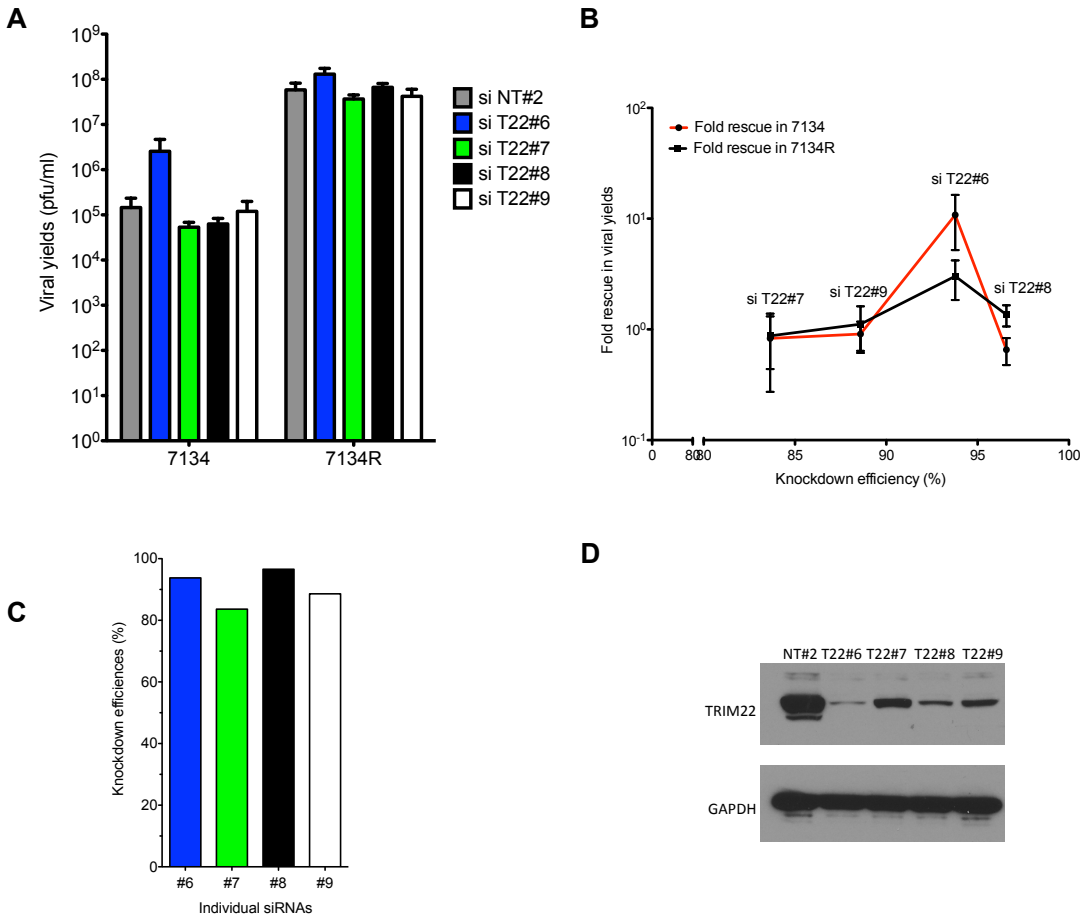


Figure 2.1 (Continued)

Figure 2.2: TRIM22 depletion by individual siRNAs increases viral yields. HFFs were transfected with individual siRNAs specific for TRIM22 (si T22#6, si T22#7, si T22#8 or si T22#9) or non-targeting siRNAs (si NT#2) and infected with HSV-1 ICP0-null (7134) or a rescued virus (7134R) at an MOI of 5. Virus yields were measured 24hpi by plaque assays on U2OS cells (n=3) (A). Virus yields were plotted against knockdown efficiencies for individual siRNAs (B). TRIM22 knockdown efficiency was measured by RT-qPCR (n=1) (C) and protein levels were determined by immunoblotting (D) at time of infection.



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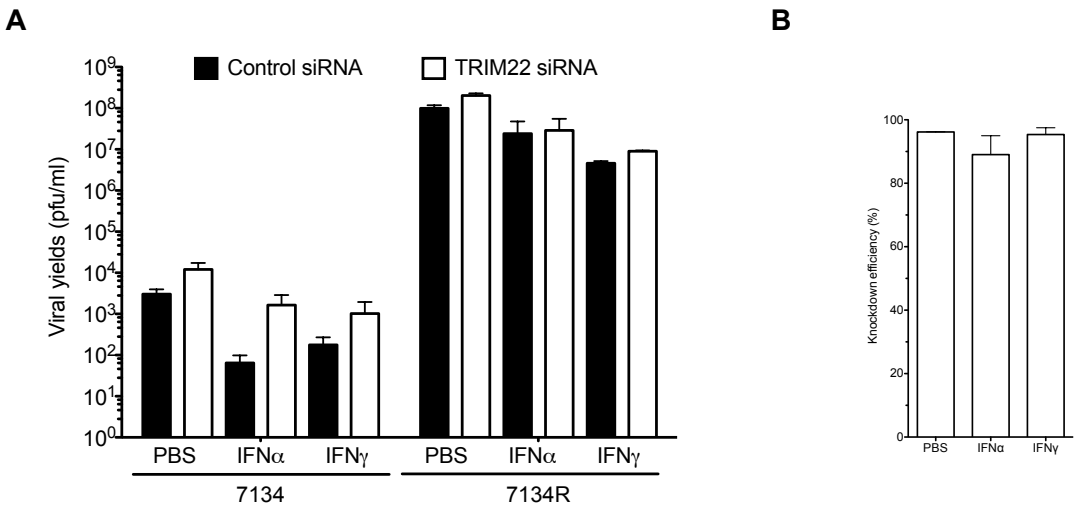

Figure 2.3: TRIM22 depletion increases virus yields in IFN pre-treated cells. HFFs were transfected with siRNA pools specific for TRIM22 or non-targeting siRNAs and were treated with PBS or hIFNα-2a (n=3) or hIFNγ (n=2) at 1000U/ml for 24h. Cells were then infected with HSV-1 ICP0-null (7134) or a rescued virus (7134R) at an MOI of 0.1 and virus yields were measured 48hpi by plaque assays on U2OS cells (A). TRIM22 knockdown efficiency was measured by RT-qPCR (n=2) (B).

Depletion of TRIM22 enhances HSV-1 IE viral gene expression

After HSV-1 enters the nucleus, the replication cycle initiates with transcription of immediate-early (IE) genes followed by early (E) gene transcription [144] . To characterize the TRIM22-mediated block in HSV-1 replication, we measured the transcript levels of IE (*ICP27*) and E (*ICP8*) viral genes in TRIM22-depleted fibroblasts relative to control-depleted fibroblasts upon 7134 and 7134R virus infection. TRIM22 depletion consistently reduced *TRIM22* transcripts relative to non-targeting siRNA transfected HFFs at 8hpi (Figure 2.4A). TRIM22 depletion increased the expression of *ICP27* and *ICP8* transcript levels by approximately 3-fold upon infection with the 7134 virus in PBS pre-treated fibroblasts at 8hpi (Figure 2.4B, C). There was also a marked increase in expression of the IE ICP4 protein, and in the E ICP8 protein levels (Figure 2.4D). We also observed a marked increase in TRIM22 protein levels in 7134 infection, consistent with TRIM22's role as an ISG (Figure 2.4D). It must be noted that the TRIM22 levels that appear lower in 7134R infection are comparable to levels observed in mock infection (Figure 2.5). These results suggest that the TRIM22-mediated inhibition of the HSV-1 ICP0-null virus is either at or prior to the level of IE gene transcription. The increase in viral IE gene expression was not due to altered nuclear entry of the virus, as TRIM22 depletion did not alter the number of 7134 virus genomes associated with the nuclear fractions at 2hpi (Figure 2.4E).

Figure 2.4: TRIM22 depletion increases viral IE, E and L gene expression. Control siRNA or TRIM22 siRNA transfected HFFs treated with PBS or hIFNα-2a at 1000U/ml for 24h were infected with HSV-1 ICP0-null (7134) or a rescued virus (7134R) at an MOI of 5. Total cell-associated RNA was harvested at 8hpi and prepared for RT-qPCR. TRIM22 transcripts (A), ICP27 transcripts (B) and ICP8 transcripts (C) were measured and normalized to 18S rRNA (n=3). Whole cell lysates were collected at 24hpi and immunoblotted for ICP4, ICP8, TRIM22 and GAPDH protein levels (D). DNA was extracted from the nuclear fractions of infected cells at 2hpi and prepared for qPCR (n=3) (E) (left panel). The western blot shows the efficiency of fractionation qualitatively (E) (right panel). Relative viral DNA levels were determined by normalizing ICP8 vDNA levels to cellular GAPDH levels. Error bars represent standard errors of the means (*P<0.05, Student's t-test).

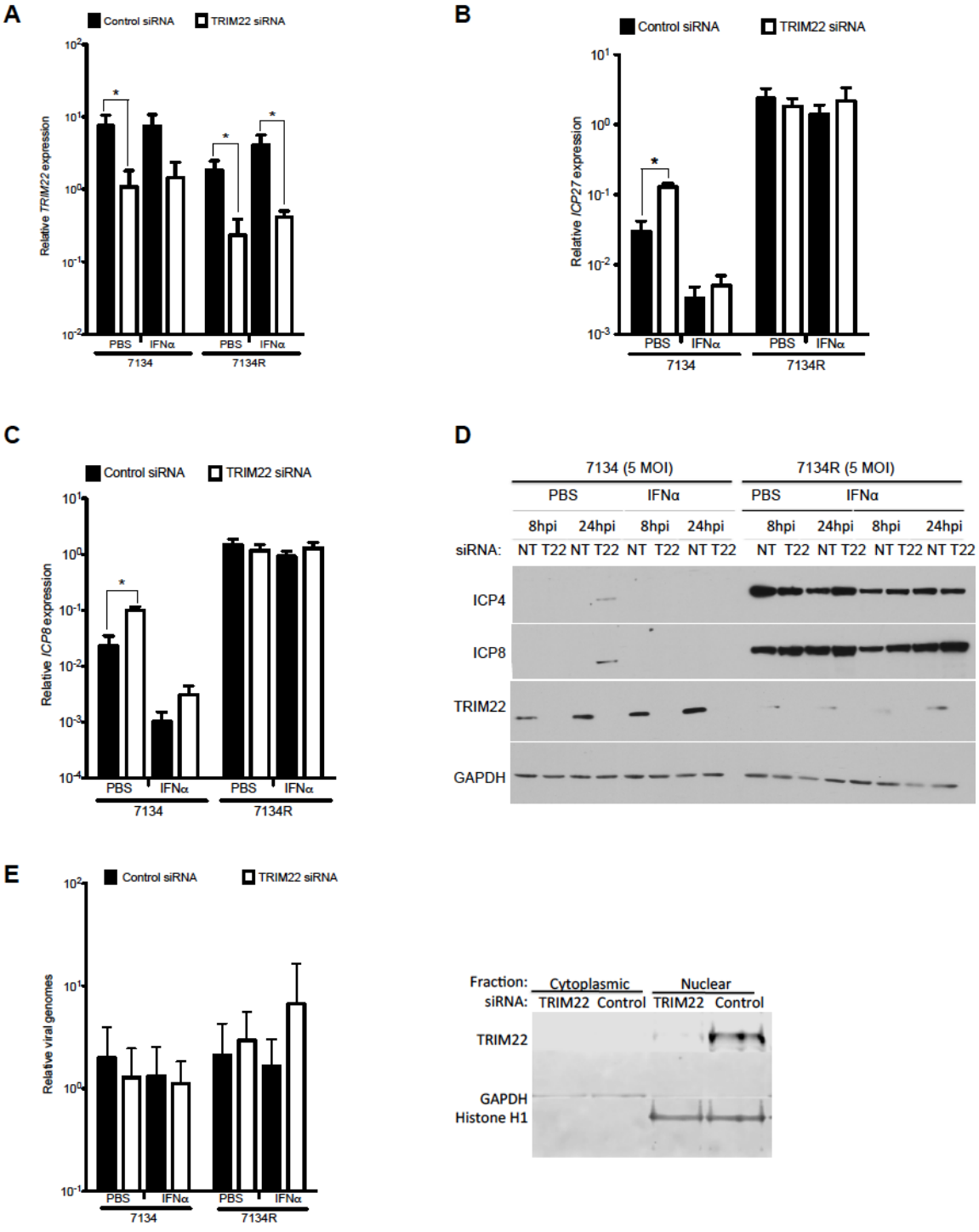

Figure 2.4 (Continued)

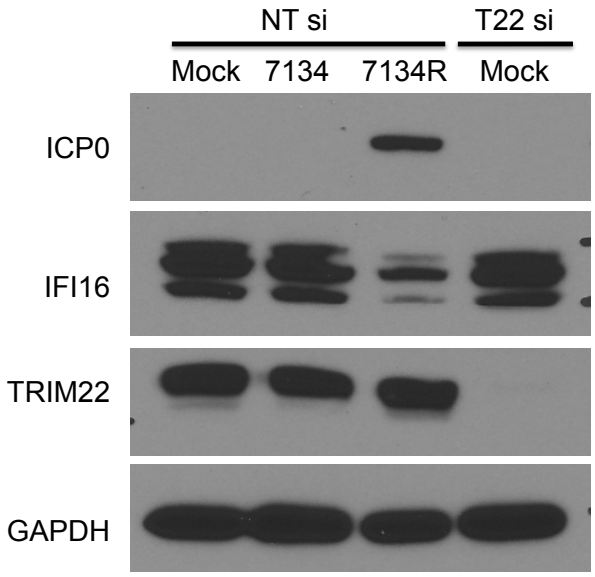

Figure 2.5: TRIM22 is not degraded by ICP0. Control siRNA (NT si) or TRIM22 siRNA (T22 si) transfected HFFs were either mock-infected or infected with HSV-1 ICP0-null (7134) or a rescued virus (7134R) at an MOI of 5. Whole cell lysates were collected at 8hpi and immunoblotted for ICP0, IFI16, TRIM22 and GAPDH protein levels.

Depletion of TRIM22 increases HSV-1 viral DNA replication and L gene expression

To determine whether the defect in IE and E gene expression was evident at the downstream stage of viral DNA (vDNA) synthesis, we measured vDNA levels in TRIM22-depleted and control-depleted HFFs. TRIM22 depletion increased vDNA synthesis of the 7134 virus in both PBS pre-treated and IFNα pre-treated HFFs, consistent with the increase in IE and E viral gene expression (Figure 2.6A). In addition, the TRIM22-mediated effect on vDNA synthesis was consistent with the increase in the expression levels of transcripts encoding components of the viral replication machinery: *UL5* (~3 fold), *UL8* (~4 fold), *UL9* (~4 fold), *UL30* (~3 fold), *UL42* (~6 fold) and *UL52* (~4 fold) (Figure 2.7). The defects in viral gene expression and vDNA replication were corroborated by L viral gene expression as TRIM22 depletion increases *gC* transcript levels and protein levels relative to control-depleted HFFs (Figure 2.8A and B). These results argued that the TRIM22-mediated defect in IE gene expression results in an inhibition of downstream viral processes, including viral replication.

Figure 2.6: TRIM22 depletion increases viral DNA replication. HFFs transfected with siRNA pools specific for TRIM22 (red lines) or non-targeting siRNAs (black lines) and treated with PBS (solid lines) or hIFNα-2a (dashed lines) at 1000U/ml for 24h were infected with HSV-1 ICP0-null (7134) (top panels) or a rescued virus (7134R) (bottom panels) at an MOI of 5. Total cell-associated DNA was harvested at 2hpi (n=4), 8hpi (n=3) and 24hpi (n=2) and prepared for qPCR (A). Relative viral DNA levels were determined by normalizing *ICP8* vDNA levels to cellular *GAPDH* levels.

A

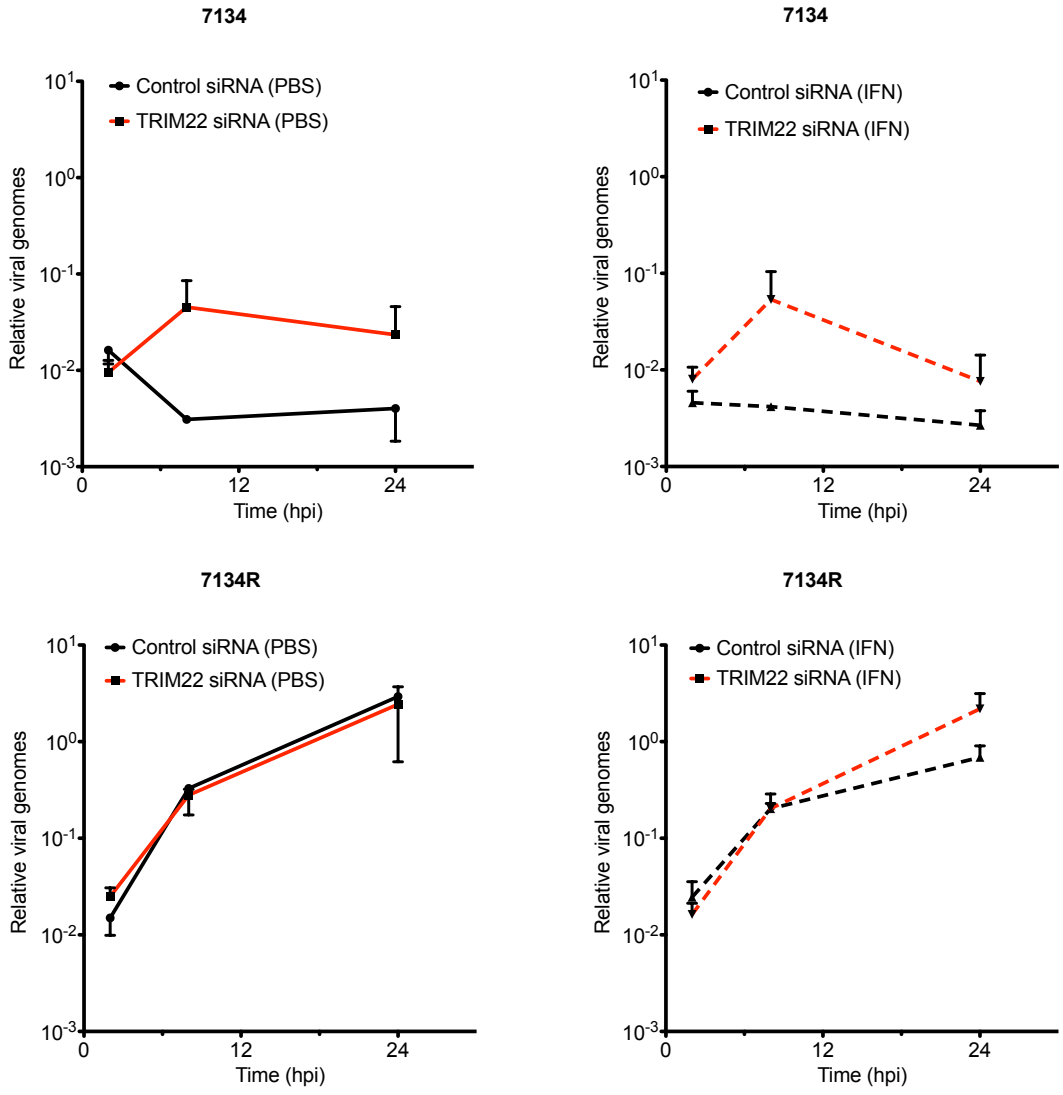

Figure 2.6 (Continued)

Figure 2.7: TRIM22 depletion increases expression of components of viral replication machinery. TRIM22-depleted or control-depleted HFFs were treated with PBS or hIFNα-2a at 1000U/ml for 24h and infected with HSV-1 ICP0-null (7134) or a rescued virus (7134R) at an MOI of 5. Total cell-associated RNA was harvested 8hpi and transcript levels of *UL5* (A), *UL8* (B), *UL9* (C), *UL30* (D), *UL42* (E) and *UL52* (F) were measured by RT-qPCR. The transcript levels were normalized to *18S* rRNA (n=3). Fold differences due to TRIM22 depletion are shown above corresponding bars.

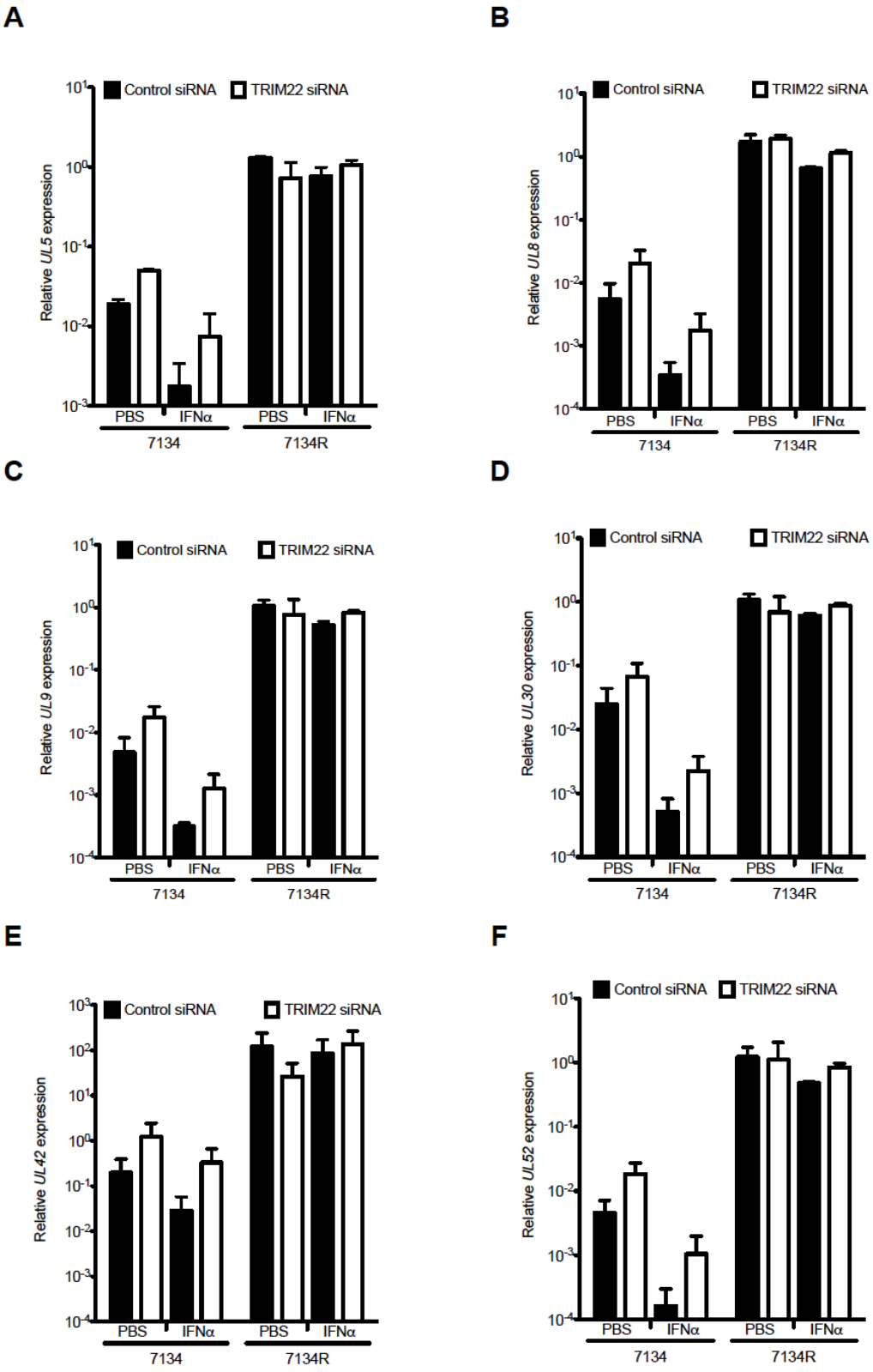

Figure 2.7 (Continued)

Figure 2.8: TRIM22 depletion increases L gene expression. HFFs were transfected with siRNA pools specific for TRIM22 or non-targeting siRNAs and were treated with PBS or hIFNα-2a at 1000U/ml for 24h and infected with HSV-1 ICP0-null (7134) or a rescued virus (7134R) at an MOI of 5. Total cell-associated RNA was harvested at 4hpi (left panel) and 8hpi (right panel) and prepared for RT-qPCR. *gC* transcripts were measured and normalized to 18S rRNA levels (n=2) (A). Whole cell lysates were collected at 4hpi and 8hpi and the representative western blot shows gC, TRIM22 and GAPDH protein levels.

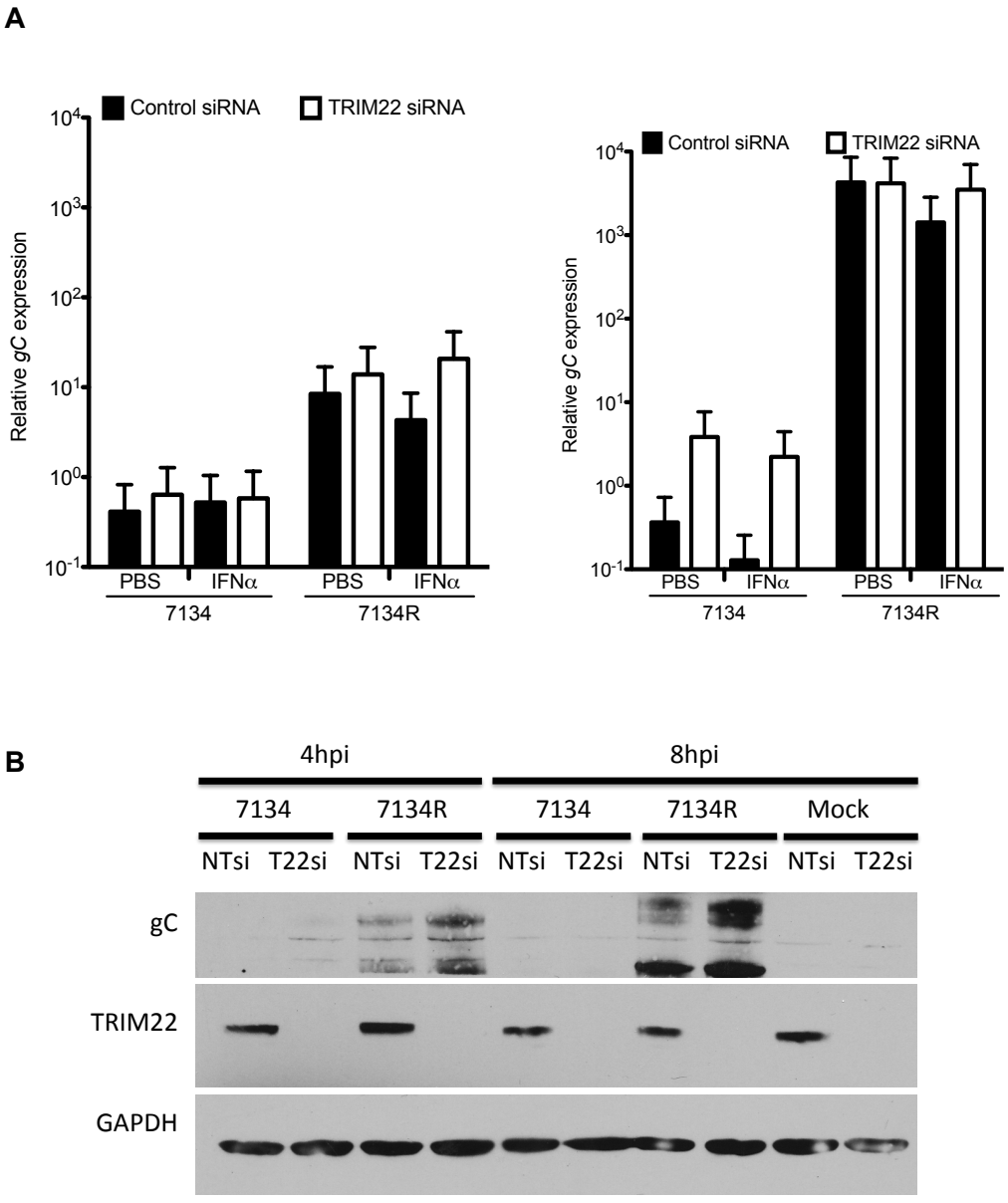

Figure 2.8 (Continued)

Over-expression of TRIM22 reduces ICP0-null and ICP0-rescued virus replication

To test whether TRIM22 was sufficient for the inhibition of the 7134 virus, we measured virus yields in HeLa cells transfected with either a *TRIM22*-encoding plasmid or a control plasmid (pLPCX). We chose HeLa cells because they express *TRIM22* at low levels relative to the other cell types that we screened and HeLa cells were also used as cell lines of choice in prior publications of TRIM22 (Figure 2.9, [142]). We observed high levels of TRIM22 transcripts and protein upon transfection relative to the empty vector (Figure 2.10A, B). TRIM22 over-expression in HeLa cells reduced both 7134 and 7134R virus yields by approximately 10-fold at an MOI of 0.1 pfu/cell relative to pLPCX transfection (Figure 2.10C). The magnitude of this difference was negligible at a high MOI of 5 in HeLa cells, despite high levels of expression of the TRIM22 transcripts and protein upon transfection (Results not shown). This MOI-dependent effect was consistent with previous studies using exogenous expression systems of the rhesus paralog, rhTRIM5α, where the TRIM5-mediated inhibition of HSV-1 is overcome at higher MOIs [135]. In addition, the exogenous expression of TRIM22 protein reduced IE (ICP4) protein levels (Figure 2.10C), complementing the results observed in RNA interference studies. Therefore, these results argued that TRIM22 is sufficient in the context of HSV-1 inhibition and that this block in replication is at the stage of IE viral gene expression.

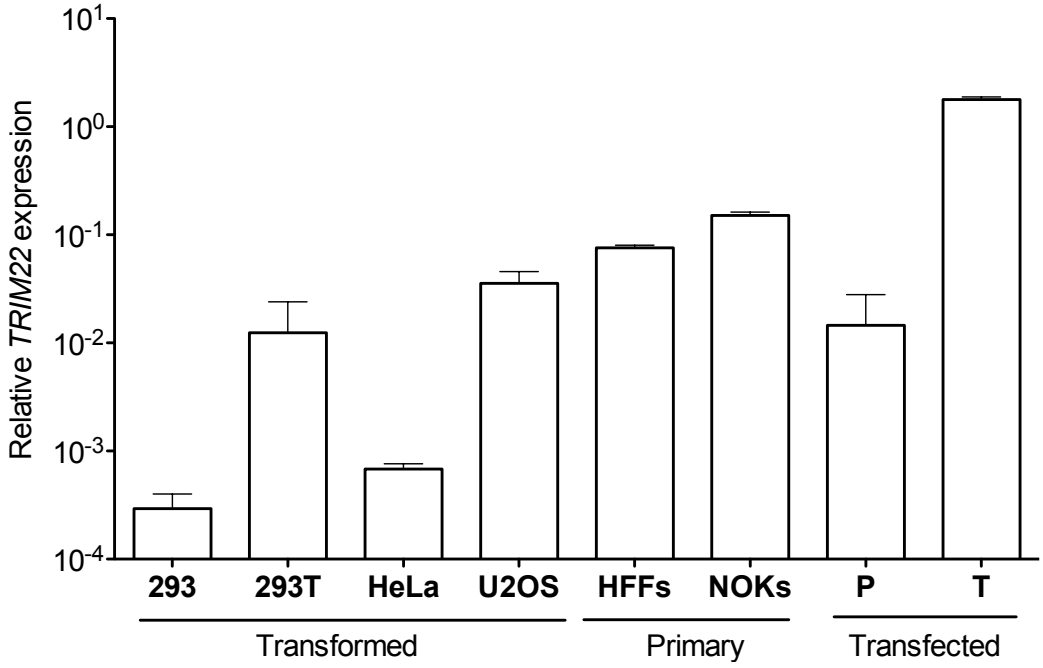

Figure 2.9: TRIM22 expression in different cell types. RNA was prepared for RT-qPCR from transformed cells (293, 293T, HeLa, U2OS) or primary cells (HFFs, normal oral keratinocytes) or HeLa cells transfected with the empty vector, pLPCX (P) or the vector encoding full-length TRIM22 (T). The levels of *TRIM22* transcripts were measured and normalized to *18S* rRNA (n=3).

Figure 2.10: Overexpression of TRIM22 reduces HSV-1 replication. HeLa cells transfected with an empty vector control (pLPCX) or a vector with a TRIM22 insert (TRIM22) were treated with PBS or hIFNα-2a at 1000U/ml for 24h. Transfected cells were infected with ICP0-null (7134) or rescued virus (7134R) at an MOI of 0.1. Total cell-associated RNA was harvested at time of infection and prepared for RT-qPCR. *TRIM22* transcripts were normalized to 18S rRNA (A). Virus yields were determined 48hpi with plaque assays on U2OS cells (B) (n=4). Representative western blot showing total cell lysates probed for ICP4, TRIM22 and GAPDH protein levels at 24hpi (C) (*P<0.05, Student's t-test).

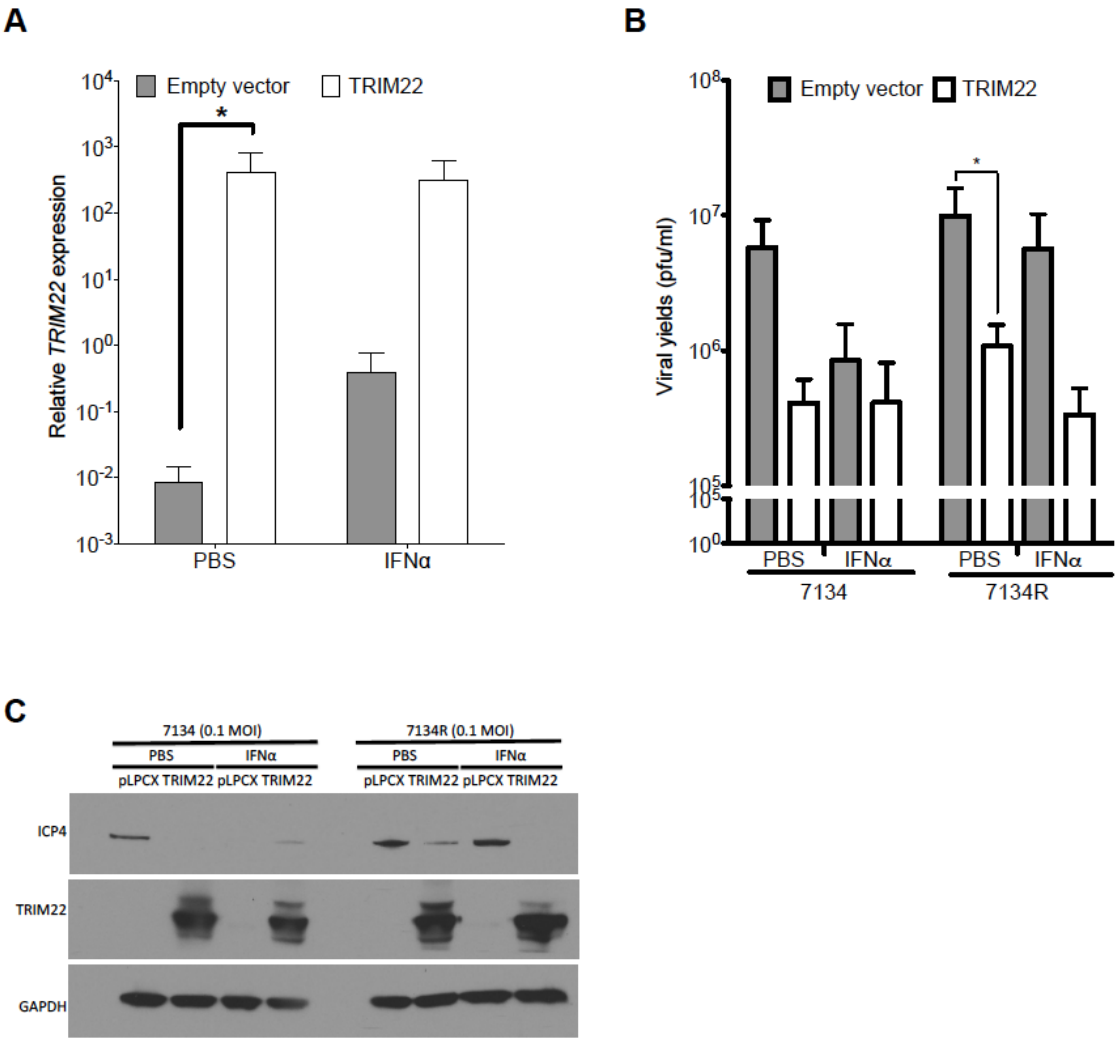

Figure 2.10 (Continued)

Inhibition of HSV-1 by TRIM22 is dependent on its nuclear localization

The domains of TRIM22 have different functions in the cell. The N-terminal RING domain has E3 ubiquitin ligase activity [89] and was reported to mediate anti-viral activity against hepatitis B virus (HBV) [145], encephalomyocarditis virus (ECMV) [109] and influenza A virus (IAV) [91]. The C-terminal B30.2/SPRY domain and the bipartite nuclear localization signal (NLS) are necessary for the nuclear localization of TRIM22 [142]. Therefore, we investigated whether these domains were necessary for TRIM22-mediated inhibition of HSV-1. To define the expression levels and localization properties of these TRIM22 mutants, we transfected HeLa cells with the following plasmids: empty vector or FLAG-tagged constructs encoding full-length TRIM22 (TRIM22), a nuclear localization signal deleted TRIM22 (TRIM22-ΔNLS), a SPRY-domain deleted TRIM22 (TRIM22-ΔSPRY), a RING-domain deleted TRIM22 (TRIM22-ΔRING) and an E3 ubiquitin-ligase inactive form of TRIM22 (TRIM22-C15A/C18A) [142]. The transfected constructs localized to sites in the cell as described previously (Figure 2.11A, [142]). The full-length TRIM22 formed nuclear punctae, as did the TRIM22-ΔRING protein and the TRIM22-C15A/C18A proteins to a smaller extent (Figure 2.11A). The TRIM22-ΔNLS localized to both the nucleus and cytoplasm, whereas the TRIM22-ΔSPRY localized to the cytoplasm (Figure 2.11A). The encoded proteins were expressed at the expected sizes and at comparable levels, except for TRIM22-C15A/C18A, which showed drastically lower protein levels consistent with the lower expression seen in immunofluorescence (Figure 2.11B).

Figure 2.11: TRIM22 nuclear localization mediates HSV-1 inhibition.

Subcellular localization in HeLa cells transfected with an empty vector control (Empty vector) or vectors encoding full-length TRIM22 (TRIM22), the nuclear localization signal deleted mutant of TRIM22 (TRIM22-ΔNLS), the SPRY-domain deleted TRIM22 (TRIM22-ΔB30.2/SPRY), the RING-domain deleted TRIM22 (TRIM22-ΔRING), the E3 ubiquitin-ligase inactive form of TRIM22 (TRIM22-C15A, C18A) was determined by staining for anti-FLAG and nuclei were counter-stained with DAPI (A). Transfected cells were lysed and probed for the FLAG-tag and GAPDH as a loading control (B). Transfected cells were infected at 0.1 MOI with 7134 or 7134R viruses and virus yields were measured 48hpi by plaque assays on U2OS cells (n=6) (C). (*P<0.05, ***P<0.001 Two-way ANOVA with Bonferroni tests).

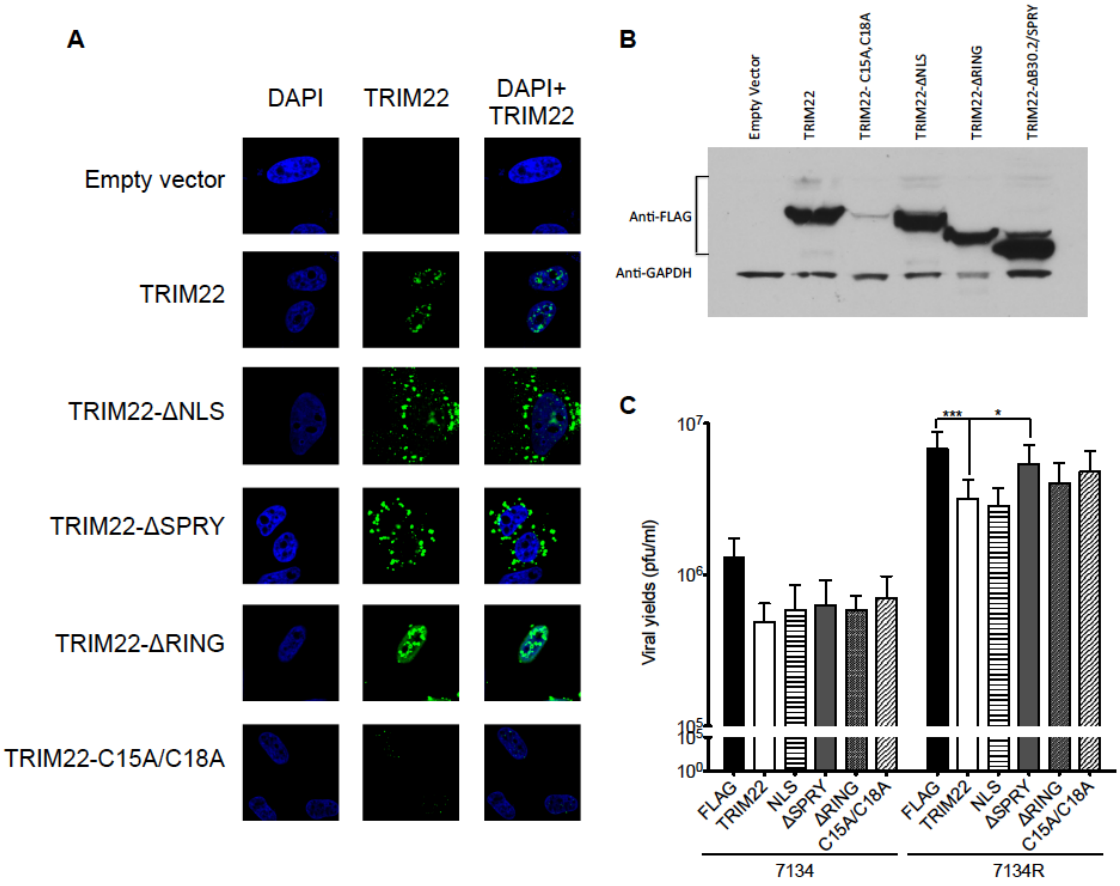

Figure 2.11 (Continued)

We infected the transfected cells with the 7134 and 7134R viruses at 0.1 MOI and measured virus yields 48hpi. Transfection with TRIM22 or any of the other TRIM22 mutants reduced viral yields of the ICP0-null virus by approximately 2-fold relative to the empty vector transfection (Figure 2.11C). Transfection with TRIM22 significantly reduced 7134R viral yields (Figure 2.11C). Interestingly, transfection with TRIM22-ΔNLS showed comparable viral yields to transfection with TRIM22 (Figure 2.11C). This is consistent with the partial localization of TRIM22-ΔNLS (Figure 2.11B). However, transfection with the SPRY domain deleted mutant (TRIM22-ΔSPRY) reduced the TRIM22-mediated inhibition to levels similar to those observed upon transfection with the empty vector (Figure 2.11C). This is consistent with the cytoplasmic localization of the TRIM22-ΔSPRY mutant (Figure 2.11B). Interestingly, transfection with the RING domain deletion mutant and the inactive RING mutant (TRIM22-ΔRING and TRIM22-C15A/C18A) showed an intermediate phenotype in reducing the TRIM22-mediated inhibition of the 7134R virus (Figure 2.11C), despite the nuclear localization of these proteins (Figure 2.11B).

These results argued that the nuclear localization conferred by the SPRY domain of TRIM22 is important for the restriction of HSV-1, as is the E3 ubiquitin ligase activity conferred by the RING domain to a smaller extent. The NLS mutant does not seem to be as functionally effective in excluding TRIM22 from the nucleus, and therefore is still capable of HSV-1 restriction.

TRIM22 depletion reduces total histone occupancy and facultative heterochromatin on IE viral gene promoters

The host cell silences viral DNA upon nuclear entry by incorporating free DNA into chromatin [146-148]. In addition, histones are post-translationally modified, thereby leading to heterochromatin formation (H3K9me3 and H3K27me3) or euchromatin formation (H3K9Ac and H3K4me3). To determine whether TRIM22 depletion increases viral gene expression due to reduced histone occupancy or by modifying histones on viral gene promoters, we conducted chromatin immunoprecipitation assays on total histone H3, the heterochromatin mark, H3K9me3, the facultative heterochromatin mark, H3K27me3, and the euchromatin marks H3K4me3 and H3K9Ac on the viral IE *ICP4* and *ICP27* gene promoters in TRIM22-depleted fibroblasts relative to control-depleted fibroblasts. TRIM22 depletion significantly reduced the total histone H3 occupancy on the viral IE gene promoters (Figure 2.12A). In addition, TRIM22 depletion reduced the density of H3K9me3 and H3K27me3 immunoprecipitated on the *ICP27* and *ICP4* promoters in the 7134 virus infection, indicating that the viral DNA is not silenced to the same extent in TRIM22-depleted fibroblasts (Figure 2.12B and C). The reduced facultative heterochromatin on vDNA in TRIM22-depleted fibroblasts was consistent with the increase in viral IE gene expression observed in Figure 2.4.

Although TRIM22 depletion did not significantly change the levels of euchromatin marks on *ICP27* and *ICP4* promoters, a greater proportion of *ICP27*

transcripts were detected with H3K9Ac immunoprecipitation (Figure 2.12D and E). These results argued that the absence of TRIM22 prevents compaction of viral DNA, reduces heterochromatin and facultative heterochromatin, providing a mechanism by which TRIM22 inhibits HSV-1 gene expression and replication.

Figure 2.12: TRIM22 depletion reduces total H3 and facultative heterochromatin association with viral DNA. Control- or TRIM22-siRNA transfected HFFs were infected with HSV-1 ICP0-null (7134) or HSV-1 ICP0-rescued (7134R) viruses at a MOI of 5. ChIP was conducted on cell extracts prepared at 6hpi with antibodies specific for histone H3 (n=6) (A), the heterochromatin mark H3K9me3 (n=3) (B), the facultative heterochromatin mark H327me3 (n=3) (C), the euchromatin marks H3K9Ac (n=3) (D) and H3K4me3 (n=3) (E). Immunoprecipitated ICP27 (left panels) and ICP4 right panels) promoter sequences were measured by qPCR and viral DNA sequences were normalized to immunoprecipitated GAPDH DNA. (*P<0.05, **P<0.01 Student's t-test).

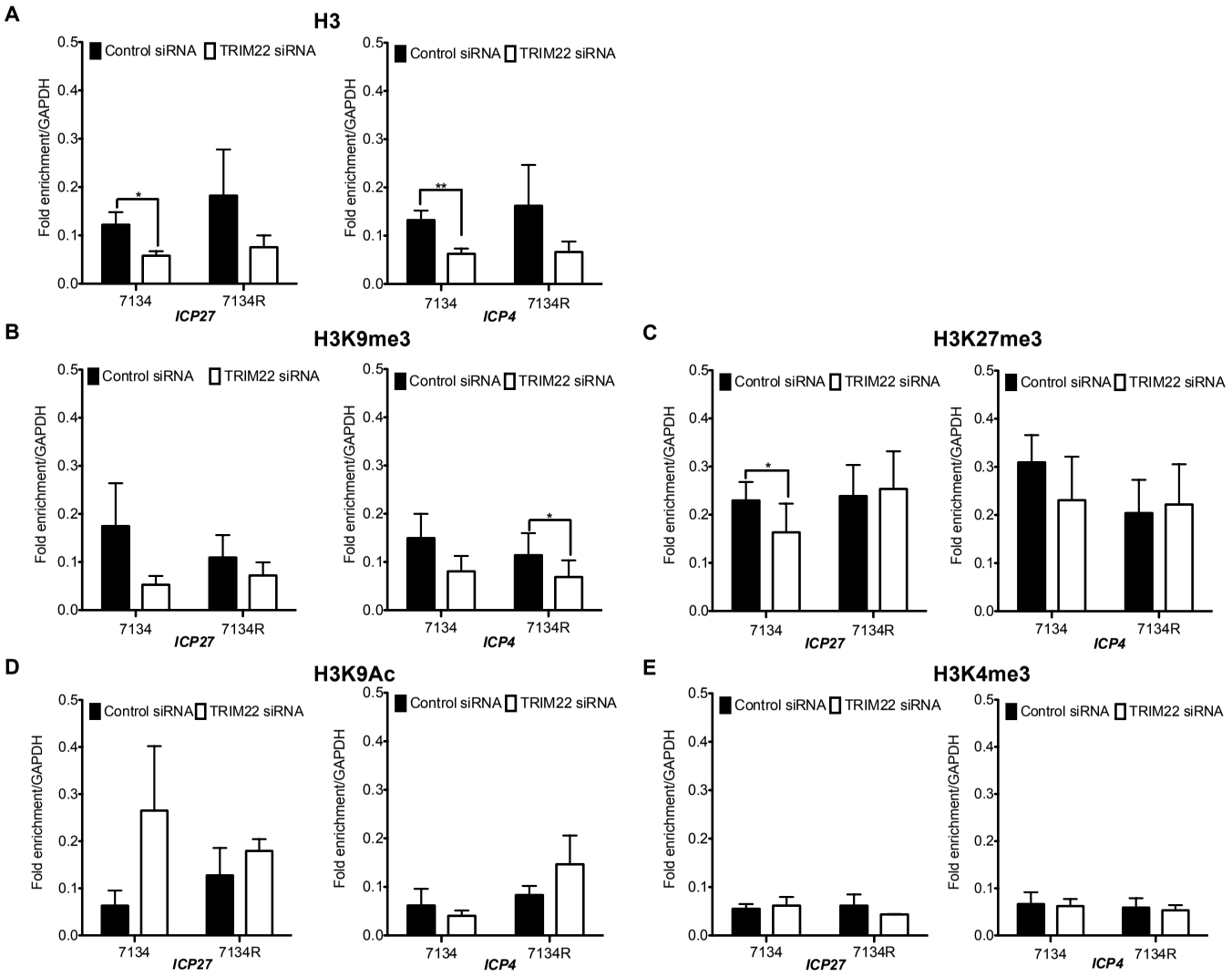

Figure 2.12 (Continued)

TRIM22 restricts a β- and a γ-herpesvirus

Previous reports demonstrated that TRIM22 inhibits a diverse range of viruses, including RNA viruses such as HIV-1, EMCV, IAV, and a DNA virus, HBV [91,94,108,109]. To test whether TRIM22 was capable of inhibiting other herpesviruses besides the canonical α-herpesvirus, HSV-1, we investigated whether TRIM22 could inhibit a γ-herpesvirus, Epstein-Barr virus (EBV). In fact, the latent membrane protein 1 (LMP1) of EBV establishes an anti-viral cellular state by up-regulating TRIM22 among other ISGs to prevent super-infection [112]. Therefore, we measured the efficiency of EBV-GFP infection in HEK293 cells expressing the EBV receptors, CD21 and HLA-II, after stable transfection with the empty vector (pLPCX) or full-length TRIM22 (TRIM22). Transformation of cells with *TRIM22* did not significantly alter the expression of CD21 (Figure 2.13A) or HLA-II (Figure 2.13B) or the percentage of $CD21^+$ $HLA\text{-}II^+$ transduced HEK293 cells (Figure 2.13C). TRIM22 expression was confirmed by immunoblotting in multiple single-cell clones and compared to endogenous levels in cells expressing the empty control pLPCX vector (Figure 2.13D). We observed significantly fewer EBV-GFP-positive cells in TRIM22-transfected cells relative to pLPCX-transfected cells (Figure 2.13E). These results argued that TRIM22 inhibits the efficiency of EBV-GFP infection. We also measured HCMV viral yields at 72hpi in control-depleted versus TRIM22-depleted HFFs. TRIM22 depletion significantly increased HCMV viral yields by approximately 2.5-fold relative to control-depletion (*P = 0.0364)* (Figure 2.13F). These results argued that TRIM22 inhibits HCMV replication also. Interestingly, TRIM22 transfection

did not inhibit the replication of an RNA virus that replicates in the cytoplasm, VSV-GFP (Figure 2.13G). In conclusion, the TRIM22-mediated viral inhibition is not limited to the α-herpesviruses and TRIM22 potentially inhibits other DNA viruses that replicate in the nucleus, including the β- and γ- herpesviruses.

Figure 2.13: TRIM22 inhibits a β- and a γ- herpesvirus. HEK293 cells stably transfected with CD21 and HLA-II, and either the empty vector or the full-length TRIM22 vector were infected with EBV-GFP at 17 Green Raji Units (GRU). CD21 MFI (A) and HLA-II MFI (B) and percentage of live cells as determined by counter-staining with aqua-amine that are CD21 and HLA-II double-positive (C) were quantified by flow cytometry. Whole cell lysates from stably transfected empty vector or TRIM22 transfected single-cell clones were probed for TRIM22 and β actin by immunoblotting (D). Percentage of live EBV-GFP cells in empty vector and TRIM22 cells were quantified 5 dpi. Matched clones are connected with lines (E) (n=4). Control- or TRIM22- siRNA transfected HFFs were infected with HCMV Ad 169 at a MOI of 1 and virus yields were measured by plaque assays on HFFs at 72hpi (F) (n=3). HEK293 cells stably transduced with either the empty vector or the full-length TRIM22 vector were infected with VSV-GFP at a range of dilutions from 0 to $2.5x10^6$ pfu/ml, and assessed by flow cytometry for live, infected cells at 16hpi (G) (n=3). (P<0.05*, Student's t-test).

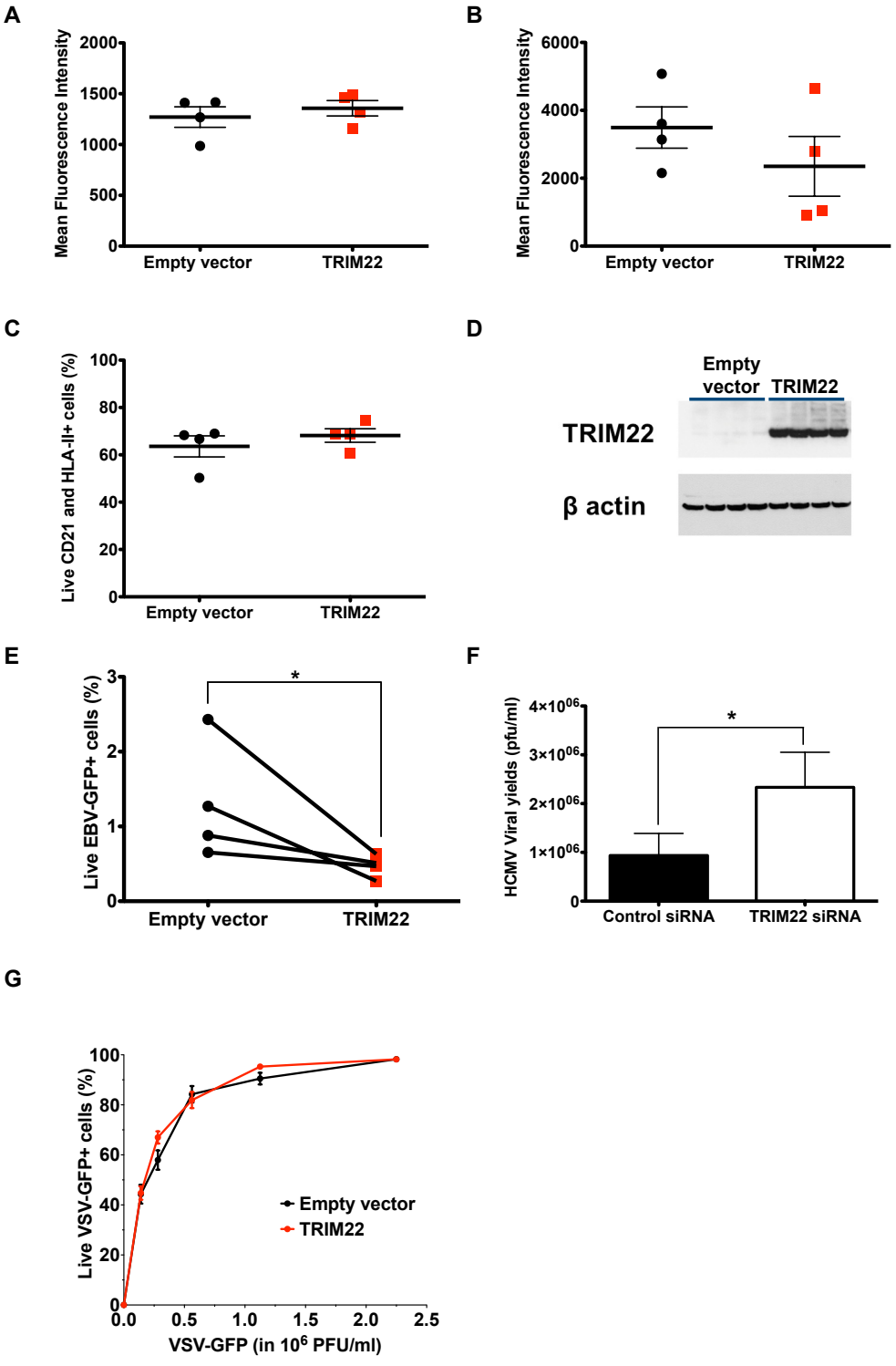

Figure 2.13 (Continued)

Discussion

TRIM22 as a restriction factor of HSV-1 by epigenetic regulation of viral genes

HSV-1 restriction in the nucleus has been previously ascribed to components of ND10 bodies in the nucleus consisting of TRIM19/PML, hDaxx, Sp100 [42,149,150], and to nuclear IFI16 [143,151]. We add TRIM22 to this list by demonstrating that TRIM22 is necessary and sufficient for restriction of HSV-1 inhibition. TRIM22 depletion in fibroblasts rescued the replication of an ICP0-l virus and to a smaller extent, an ICP0+ virus. The inhibition of both the ICP0- and ICP0+ viruses in TRIM22 over-expression studies in HeLa cells further corroborates this finding. Interestingly, the magnitude of TRIM22-mediated inhibition of HSV-1 in HeLa cells is smaller at high MOIs, reflecting a MOI-dependent effect where the virus is capable of overcoming the intrinsic restriction at high MOIs in HeLa cells. This MOI-dependent effect on viral restriction is a common feature of intrinsic immunity and was previously reported in the rhesus macaque TRIM5**α** mediated restriction of HIV-1 [73,152].

TRIM22 was previously shown to inhibit viruses by various mechanisms, including degradation of viral proteins, as seen in EMCV [109] and IAV [91], transcriptional repression [107,108] and inhibition of viral assembly [94], as seen in HIV-1, and prevention of viral core promoter activity, as seen in HBV [110]. We discovered that the TRIM22-mediated inhibition of HSV-1 replication was due to a defect in viral IE gene expression after nuclear entry of viral genomes, with downstream effects on E gene expression, viral DNA synthesis and L gene

expression. The mechanism of inhibition is novel in that TRIM22 depletion reduces histone occupancy and heterochromatin marks on viral DNA; chromatin modification as a means of viral inhibition has been directly attributed to IFI16 [143], and ATRX in concert with hDaxx to a smaller extent, as a chromatin remodeling complex [42]. The significance of this finding is implicated in a review by Hattlman, CJ [153] who hypothesized that TRIM22 could contribute to viral latency based on the observations that (i) the EBV Latent Membrane Protein 1 (LMP1) required for EBV latency upregulates TRIM22 [112] and (ii) KSHV LANA protein upregulates TRIM22 *in vitro* and in KSHV lesions [111]. In both cases, TRIM22 was upregulated by viral proteins associated with latency and this was proposed to prevent super-infection by other viruses [111,112]. However, a mechanism for this connection to latency has not been determined. Thus, it was of interest to us that TRIM22 epigenetically regulates viral gene expression by the observed decrease in histone occupancy in the absence of TRIM22 and the reduction in heterochromatin and facultative heterochromatin marks on viral gene promoters in lytic infection. Further studies need to be done on whether TRIM22 can contribute to viral latency by modifying chromatin.

TRIM22 restricts viruses that replicate in the nucleus

The importance of nuclear-specific inhibition of HSV-1 was evident from PML, Daxx, Sp100 and ATRX in ND10 bodies [42,150], and IFI16 [143], which are crucial for nuclear-specific inhibition of HSV-1. In addition, the endogenous nuclear localization of TRIM22 in primary fibroblasts, where the inhibitory effect

of TRIM22 was observed, lends further evidence to the case for recognition and restriction of foreign DNA in the nucleus. This report expands the anti-viral specificity of TRIM22 to include viruses with dsDNA genomes that replicate in the nucleus, the herpesviruses.

As mentioned previously, the TRIM22-mediated anti-viral mechanisms of inhibition of other viruses can be predominantly attributed to its E3 ubiquitin ligase activity located in the N-terminal RING domain of the protein [91,109,110]. In the case of HSV-1, the formation of nuclear punctae seems equally as important as the E3 ubiquitin ligase activity of TRIM22 for the anti-viral effect, as the absence of the RING domain or the inactive RING-domain mutant, TRIM22-C15A, C18A only partially alleviated the TRIM22-mediated restriction of HSV-1. In contrast, the deletion of the SPRY domain significantly reduced TRIM22-mediated restriction and therefore this domain is also required for mediating the antiviral activity.

In fact, the SPRY domain of TRIM22 has been implicated in the protein's subcellular localization as evidenced by the following key observations in the literature: (i) The absence of B30.2/SPRY domains in a transfection system prevents the formation of nuclear punctae [142], (ii) B30.2/SPRY domain swaps between human and rhesus TRIM22 proteins alters the subcellular localization of these proteins [154]. In addition, the importance of the SPRY domain was also evident in TRIM22's anti-viral activity, in that, the absence of the SPRY domain was shown to abrogate the inhibition of HBV [110]. Interestingly, the construct lacking the SPRY domain localized primarily to the cytoplasm, in a location

different from the nuclear-replicating HBV [110]. The phenotype of the ΔSPRY domain mutant in HSV-1 infection is similar. These results are suggestive of the importance of nuclear localization or other intranuclear activity of TRIM22 in the inhibition of viruses that replicate in the nucleus, such as other herpesviruses.

Therefore, TRIM22 inhibits viruses that replicate in the nucleus: IAV [91], HBV [110], and in this study herpesviruses including members of the α, β and γ *Herpesviridae*, while it is unable to restrict viruses that replicate in the cytoplasm such as VSV, a RNA virus. On the other hand, TRIM22 also inhibits EMCV, a positive strand RNA virus that replicates in the cytoplasm [109]. However, these studies were conducted in transient transfection settings where the protein can potentially show cytoplasmic localization and suggest that TRIM22 may also have a role in the cytoplasm. This is consistent with the activity of other restriction factors that localize to and inhibit pathogens in particular compartments.

HSV-1 mediated escape of TRIM22-mediated inhibition

We attribute the ability of the wild-type virus to overcome TRIM22-mediated inhibition to the viral IE protein, ICP0. ICP0 is crucial to alleviating a majority of the intrinsic- and the Type I IFN mediated- immune responses: i.e., (i) it disrupts PML bodies at sites of viral replication [155], preventing their intrinsic inhibition; (ii) it sequesters phosphorylated IRF3 from the nucleus [128,156], thus preventing its activity as a transcription factor and (iii) it degrades the nuclear viral DNA sensor and intrinsic resistance factor, IFI16, thus preventing detection

and silencing of viral DNA [30]. Therefore, the finding that ICP0 rescued most of the intrinsic block by TRIM22 is consistent with previous studies. However, this reduction in inhibition is not due to the ICP0-mediated degradation of TRIM22, as viruses with ICP0 such as the 7134R and the *d106* viruses did not degrade TRIM22 (Figure 2.5, Results not shown). Surprisingly, TRIM22 is not degraded by ICP0, and it remains to be determined how the virus evades the intrinsic restriction activity of TRIM22 and whether there are other viral factors that antagonize TRIM22.

In conclusion, the key features of restriction factors are that they (i) are germline-encoded, expressed constitutively, and often interferon (IFN)-inducible; (ii) demonstrate atypical mechanisms of viral inhibition and; (iii) show hallmarks of positive genetic selection (high d_N/d_S ratios) reflecting host-pathogen co-evolution [39]. Consistent with previous reports, we show that TRIM22 is IFN-inducible, restricts HSV-1 in an epigenetic fashion, and was demonstrated to have a high dN/dS ratio by other laboratories [116]. Thus, we report that TRIM22 is a novel restriction factor of HSV-1. We also report that this restriction is mediated by epigenetic silencing of viral gene expression, and the detailed mechanisms of TRIM22 mediated repression of viral DNA remain to be further explored.

Chapter 3:

TRIM22 HAPLOTYPIC VARIATION ALTERS SUSCEPTIBILITY TO HERPESVIRUSES

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Author contributions: T.S.R., N.L.L., and D.M.K., designed research; T.S.R., P.E.M. and T.R. performed research; S-Y.L. identified haplotypes, generated constructs and stably transduced cell lines for haplotypes; T.S.R. and D.M.K. analyzed data; T.S.R. and D.M.K. wrote the paper.

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*Deceased

Abstract

Variation in genes encoding restriction factors, such as non-synonymous single nucleotide polymorphisms, can alter the anti-viral functionality of those proteins. The genetic variation in the *TRIM5* and *TRIM22* gene neighbors was previously attributed to the lentiviruses, such that these two genes were mutually selected for in different primate lineages throughout evolution. Our collaborators characterized novel variation at the *TRIM22* locus, or seven *TRIM22* haplotypes. They identified four single nucleotide polymorphisms encoding non-synonymous amino acid substitutions in the linker L2 and coiled-coil domains of TRIM22. We demonstrate that the asparagine to aspartic acid amino acid substitution at position 155 in the coiled-coil domain of TRIM22 reduce correlates of EBV infection *in vitro*, *in silico* and *in vivo*. We also show that the threonine to arginine substitution at position 242 in the linker L2 domain reduce HSV-1 viral yields *in vitro*. Therefore, we hypothesize that the haplotypic diversification observed in *TRIM22* is an example of the host-virus arms race in primate evolution, and propose that the *TRIM5* and *TRIM22* gene neighbors evolved due to selective pressure from both herpesviruses and lentiviruses.

Introduction

Non-synonymous single nucleotide polymorphisms (nsSNPs) in genes alter not only the amino acid composition of the proteins they encode, but can also change their structure and function. In fact, approximately 50% of inherited genetic diseases have mutations associated with nsSNPs [157-159]. Mutations in innate immune genes have also been associated with a variety of inflammatory disorders, including asthma [160], tuberculosis [161], and many others (reviewed in [162]).

Genetic variation in host innate immune proteins can also alter the host's susceptibility to pathogens such as viruses. For instance, many of the genetic variations in the TRIM family of proteins have been shown to affect their antiviral specificity. In particular, amino acid substitutions in the coiled-coil, B-box, linker L2 and the C-terminal B30.2/SPRY domains in both the TRIM5 and TRIM22 proteins have been shown to affect the structure, function, and antiviral specificity of these proteins [55,78-82,91,92,108-110,142,163,164]. This genetic variation observed in the *TRIM5* and *TRIM22* gene loci has been attributed to evolutionary pressures exerted by retroviruses [114,116].

Amino acid substitutions in the coiled-coil and linker L2 domains in TRIM5α from rhesus macaque monkeys (rhTRIM5α) have been shown to alter its antiviral activity [165]. This is possibly because the coiled-coil domain of rhTRIM5α is important for higher-order multimerization and mediates its specificity to binding lentiviral capsids [73,165,166]. Additionally, variation in the B30.2/SPRY domain of TRIM5α has been shown to change its activity against

HIV-1 and herpesviruses [55,75,77,82,86,114,167-175]. Prior work conducted in the Letvin laboratory demonstrated that B-lymphoblastoid cell lines (B-LCLs) of rhesus macaque monkey origin expressing *TRIM5* haplotypes encoding the wild-type B30.2/SPRY domain, were significantly more permissive to β-herpesvirus, rhesus cytomegalovirus (rhCMV), and γ-herpesvirus, rhesus lymphocryptovirus (rhLCV), infections than B-LCLs encoding *TRIM5* genes with a 2-amino acid deletion in the B30.2/SPRY domain [86]. These *in vitro* differences correlated with an *in vivo* study measuring antibody titers against rhCMV and rhLCV [86]. However, no significant differences in the antiviral activity of the *TRIM5* haplotypes were observed with two other DNA viruses, adenovirus 5 (Ad5) and simian vacuolating virus 40 (SV40), suggesting that this *TRIM5* haplotypic variation in anti-viral activity is specific to the rhesus macaque herpesviruses [86]. Other important genetic variations in *TRIM5* include polymorphisms that result in H43Y, R136Q, and G249D amino acid substitutions that confer variability to HIV-1 acquisition and disease progression in human patients [84,176-178].

The closest human paralog of *TRIM5* is *TRIM22 [116].* The *TRIM22* gene locus has undergone episodic positive evolutionary selection, as evidenced by a positive *dN/dS* ratio in Old World Monkeys, and hominoids including humans [116]. This positive selection resulted in *TRIM22* variation at the level of SNPs, which have been shown to be associated with functional anti-viral significance [179]. The characterized variation in *TRIM22* that alters its antiviral function include the following SNPs: rs10838543 T/C (position 364), rs7935554 A/G

(position 463) and rs1063303 C/G (position 242) [180]. For example, HIV-1 replication was higher in PBMCs from human donors homozygous at SNPs rs7935564 G/G and rs1063303 G/G [164]. In contrast, the TRIM22 SNP rs1063303 C/C decreased TRIM22's antiviral function against HIV-1 [181]. Furthermore, the TRIM22 rs10838543 C/C genotype in the Han-Chinese population is associated with chronic HBV infection [182].

Further predicted SNPs within the *TRIM22* gene were identified by *in silico* analyses across multiple databases [163]. A summary of the nsSNPs encoding the amino acid substitutions that are predicted to be functionally important follow: L68R and H73R in the linker L1 region, E135K in the coiled-coil region, I234N and S244L in the linker L2 region, G346S, K364N, P403T, K364N, P403T, L432W, R442C, F456I, T460I, P484S and C494F in the B30.2/SPRY domain [163]. Although this study demonstrated that the amino acid substitutions disrupt the structure of the TRIM22 B30.2/SPRY domain, no functional characterization was conducted on these amino acid substitutions.

These studies provide a strong evidence for the functional role and the importance of genetic variations in the genes encoding host viral restriction factors like the TRIM proteins. Here, we report here that the haplotypic variation in the *TRIM22* gene locus as identified by our collaborators [183], altered the anti-herpesviral activity of TRIM22 in *in vitro* replication studies. We further hypothesize that the haplotypic variation in *TRIM22* correlates with herpesviral loads in humans. Consequently, we speculate that the genetic variation observed

at the *TRIM22* gene locus is due to evolutionary pressure exerted by herpesviruses, in addition to the previously known retroviruses.

Materials and Methods

Cell Culture. HEK293, HeLa and CF2TH cells were obtained from the American Type Culture Collection (ATCC). HEK293 cells stably transduced with CD21 and HLA-II were obtained from the Fingeroth laboratory and cultured as described previously [138]. CF2TH cells stably transduced with human *TRIM22* genes were obtained from the Letvin laboratory at Harvard Medical School and cultured in DMEM with 10% FBS and 4ug/ml puromycin [183].

Virus Infections. EBV-GFP was diluted in DMEM with 10% FBS and Pen-Strep. EBV-GFP stocks were prepared from a cell line kindly donated by Wolfgang Hammerschmidt as described previously [141]. The virus was diluted in DMEM containing 10% FBS and the cells were infected for 5 days at 37°C. VSV-GFP was used according to described protocols and was a kind gift from Sean Whelan [184].

Plasmids and DNA Transfection. The TRIM22 cDNA was extracted from B lymphoblastoid cell lines from the Hap Map project and cloned into the pLPCX vector backbone (Clontech). HeLa cells were transfected with Effectene reagent and then infected at 24h after transfection. CF2TH cells stably transduced with the different TRIM22 haplotypes are a kind gift from So-Yon Lim.

Western Blots. Cells were lysed in NuPAGE LDS Sample Buffer, and proteins were resolved on NuPAGE 4% to 12% Bis-Tris gels (Invitrogen) [143]. Proteins

were transferred overnight to nitrocellulose membranes and blocked with 5% milk in phosphate buffered saline (PBS) solution containing 0.1% Tween-20 (PBS-T). Membranes were probed with primary antibody at 4°C overnight, washed with PBS-T and incubated in secondary antibody for 1h at room temperature. Western blots were developed using the Super Signal Pico Chemiluminescence substrate.

Flow Cytometry. HEK293 cells infected with EBV-GFP or VSV-GFP were trypsinized, collected by centrifugation and resuspended in solution containing Aqua-Amine (Life Technologies). Cells were washed twice in PBS and resuspended in 2% paraformaldehyde for 15 minutes at 4°C. The cells were analyzed using a LSR II flow cytometer and live cells were gated for using an Aqua-Amine negative gate after exclusion of doublets on forward and side scatter axes. GFP+ gate was defined on mock-infected cells. Data analysis was performed using FlowJo (version 8) software and graphs were constructed by using Graph Pad Prism software.

***In silico* analyses of EBV genome copy numbers.** Frequencies of TRIM22 SNPs in HapMap B-LCLs were obtained from the dbSNP (Short Genetic Variations) from the NCBI database, using the specific rsSNP IDs assigned to different single nucleotide polymorphisms (http://www.ncbi.nlm.nih.gov/SNP/). The B-LCLs in the HapMap consortium originate from 4 different ethnic groups: a cohort of 30 trios (90 individuals) from Yoruba in Ibadan, Nigeria (YRI); a cohort of 30 trios from Utah with Northern and Western European ancestry (generated

at the Centre d'Etude Polymorphisme, CEU); from a cohort of 45 Japanese in Tokyo, Japan (JPT) and a cohort of 45 self-identified Han Chinese in Beijing, China (HCB) [180]. EBV genome copy numbers in B-LCLs were obtained from the Broad institute website (http://www.broadinstitute.org/mpg/pubs/hapmap_cell_lines/). All graphs were constructed using Graph Pad Prism software.

Antibody titers. Plasma samples from a cohort of 45 Malawi patients were titered on anti-EBV VCA IgG ELISA kits from Abcam (ab108730). Briefly, five 10-fold dilutions of plasma were added to ELISA plates pre-coated with Epstein-Barr antigen, followed by washing with phosphate buffered saline (PBS). Anti-human IgG labeled HRP was added to the plates. A colorimetric assay was conducted following catalysis of blue tetramethylbenzidine (TMB) to a yellow acidic solution after adding the stop solution. Controls included were a standard curve, EBV IgG cutoff control, EBV IgG negative control and EBV IgG positive control. The antibody absorbances were measured at OD450nm using an ELISA plate reader.

Results

The TRIM22 linker L2 domain mediates variation in susceptibility to HSV-1.

Previous studies showed that non-synonymous amino acid substitutions in the TRIM22 protein reduced its ability to inhibit HIV-1, and individuals homozygous for CC at rs10838543 in the RING domain of *TRIM22* were associated with chronic Hepatitis B virus (HBV) infection [163,164,181,182]. In order to characterize the genetic variation in *TRIM22*, our collaborators sequenced the *TRIM22* gene loci in B-LCLs generated from the Caucasian and Yoruba cohorts in the HapMap project [180]. They identified seven *TRIM22* haplotypes in these populations with the following nsSNPs encoding amino acid substitutions: rs7935564 A/G (N155D), rs1066303 G/C (T242R), rs61735273 C/T (S244L), rs730404240 C/A (T294L) (Figure 3.1A) [183]. The resultant amino acid substitutions are located in the coiled-coil domain and in the linker L2 domain of the TRIM22 protein (Figure 3.1B).

To characterize whether the *TRIM22* haplotypic variation alters susceptibility to HSV-1, we generated CF2TH cells or canine thymocyte cell lines, stably transduced with either an empty vector or each of the seven different *TRIM22* haplotypes. We infected these cells with HSV-1 7134R at a MOI of 0.1 and assessed viral yields at 48 hpi. We observed that *TRIM22* haplotypes 5, 6 and 7 all reduced 7134R yields relative to empty vector transfection, but *TRIM22* haplotype 6 showed a significant reduction in viral yields (Figure 3.1C). When we measured the level of protein expression of the transfected *TRIM22* haplotypes, we observed that haplotypes 6 and 7 were expressed at lower levels than the

rest (Figure 3.1D). Despite their lower level of expression, haplotypes 6 and 7 decreased viral replication, arguing that these *TRIM22* haplotypes are potentially more restrictive to HSV-1 infection *in vitro* relative to the rest of the haplotypes.

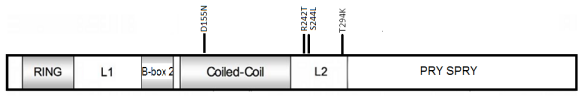
To confirm the *TRIM22* haplotype-mediated variability in HSV-1 susceptibility in another system, we transfected HeLa cells with either the empty vector or the vectors encoding each of the different *TRIM22* haplotypes. We then infected the cells with HSV-1 7134R at a MOI of 0.1 and measured viral yields at 48 hpi. Consistent with the results from the CF2TH cells, HeLa cells transfected with *TRIM22* haplotypes 5, 6 and 7 showed lower viral yields relative to the empty vector transfected cells (Figure 3.1E). It must be noted that the level of restriction observed in HeLa cells was lower compared to the CF2TH cells, possibly due to the transient transfection system in HeLa cells versus the stable transduction in CF2TH cells (Figure 3.1E). *TRIM22* haplotypes 6 and 7 showed lower levels of protein expression in HeLa cells relative to the other haplotypes consistent with the expression levels seen in the CF2TH cells (Figure 3.1F). Together, these results demonstrate that *TRIM22* haplotypes 6 and 7 encoding the amino acid arginine at position 242, amino acid leucine at position 244 and threonine at position 294 are more restrictive than other haplotypes. In addition, these results suggested that variation in the *TRIM22* gene locus could play a role in the susceptibility to HSV-1 infection in humans.

Figure 3.1: *TRIM22* haplotypes alter susceptibility to HSV-1. Full-length TRIM22 cDNAs were prepared from the human lymphoblastoid cell lines (B-LCLs) in the Caucasian and Yoruba cohorts generated in the HapMap project and sequenced. The resulting *TRIM22* haplotypes were labeled in descending order of frequency in the whole population (A). The schematic diagram shows the location of the amino acid substitutions when mapped to the predicted domain structure of the TRIM22 protein (B). CF2TH cells stably transduced with an empty pLPCX vector control (EV) or the pLPCX vectors with *TRIM22* haplotype insert (1, 2, 3, 4, 5, 6 and 7) were infected with HSV-1 7134R at 0.1 MOI. Viral yields were measured by plaque assays at 48hpi (n=3) (C). Representative western blot showing whole cell lysates probed for TRIM22 and GAPDH levels (D). HeLa cells transfected with an empty pLPCX vector control (EV) or the pLPCX vector with *TRIM22* haplotype insert (1, 2, 3, 4, 5, 6 and 7) were infected with HSV-1 7134R at an MOI of 0.1. Viral yields were determined at 48 hpi by a plaque assay on U2OS cells, (n=5) (E). Representative western blot showing total cell lysates probed for TRIM22 and GAPDH protein levels (F).

A

	dbSNP	rs7935564	rs1063303	rs61735273	rs73404240
	nt position	463	725	731	881
	nt change	AAT/GAT	ACG/AGG	TCG/TTG	ACA/AAA
	aa position	153	242	244	294
	aa change	N/D	T/R	S/L	T/L
Alleles	1	N	T	S	T
	2	**D**	T	S	T
	3	N	**R**	S	T
	4	N	T	S	**K**
	5	**D**	**R**	S	T
	6	N	**R**	**L**	T
	7	**D**	**R**	**L**	T

B



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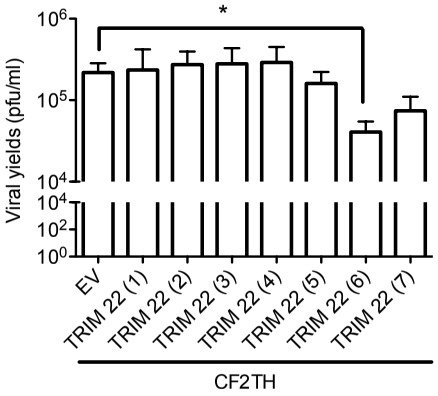

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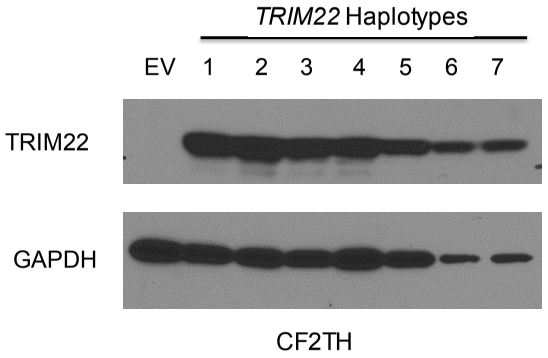

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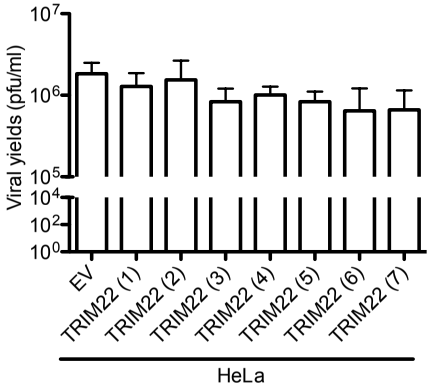

F

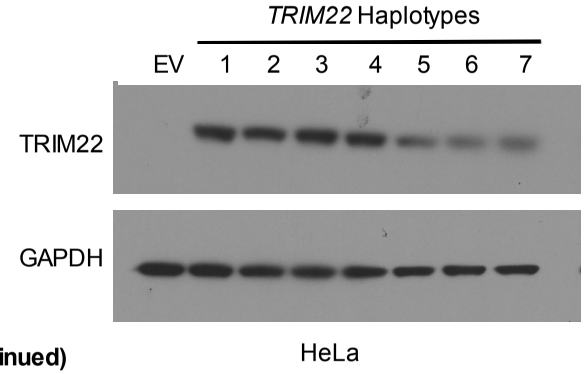

Figure 3.1 (Continued)

TRIM22 coiled-coil domain mediates variation in susceptibility to EBV.

Our collaborators and others described a nsSNP in TRIM22 encoding an N155D amino acid substitution in the coiled-coil domain [180,183]. Given that the coiled-coil domain has been shown to be important for higher-order multimerization and antiviral activity in some TRIM proteins, we wanted to identify whether the SNP encoding an N155D amino acid substitution in the TRIM22 coiled-coil domain altered the antiviral activity of TRIM22 [165]. We stably transduced CD21- and HLA-II-expressing HEK293 cells with full-length *TRIM22* haplotypes encoding either an asparagine (TRIM22 N155) or an aspartic acid (TRIM22 D155) at position 155, or with a control pLPCX vector, and assessed the efficiency of a γ-herpesvirus, or EBV infection. The cell lines were infected with EBV-GFP at MOIs up to 17 green Raji units/ml [185]. Both TRIM22 N155- and TRIM22 D155- transduced cell lines demonstrated fewer EBV-GFP$^+$ cells than the empty control vector-transduced HEK293 cells (Figure 3.2A). Moreover, TRIM22 D155-transduced HEK293 cells demonstrated consistently fewer EBV-GFP$^+$ cells compared to HEK293 cells expressing TRIM22 N155 at (Figure 3.2A). Lysates prepared from the *TRIM22*-transduced cells showed equivalent TRIM22 expression levels by immunoblotting (Figure 3.2B). Transduction of the *TRIM22* constructs into the HEK293 cells did not alter EBV receptor levels, CD21 and HLA-II, based on comparison of three separate clones of transduced cell lines (Figure 3.2C). In contrast, similar percentages of VSV-GFP$^+$ cells were observed in HEK293 cell lines transduced with either of the TRIM22 variants or the empty pLPCX control vector (Figure 3.2D). These results indicated that modification of

an N to a D at position 155 in the coiled-coil domain of TRIM22 produces a small, but consistent decrease in the efficiency of EBV infection *in vitro*. These results also suggest that this amino acid residue may play a role in the anti-viral activity of TRIM22 by altering potential multimerization of TRIM22, as has been demonstrated for rhTRIM5α [94,165,186,187].

Figure 3.2: The N155D amino acid substitution in TRIM22 decreases the efficiency of EBV-GFP infection in vitro. HEK293 cells stably expressing CD21 and HLA-II were transduced with TRIM22 constructs encoding an asparagine at amino acid position 155 (TRIM22 N155, filled circles) or TRIM22 encoding an aspartic acid at position 155 (TRIM22 D155, filled squares) or the empty pLPCX vector (hollow diamonds). Cells were infected in triplicates with an EBV-GFP construct for 48h and live, GFP^+ cells were measured by flow cytometry (A). The expression of TRIM22 protein by the transduced cells was determined by immunoblotting using an anti-TRIM22 antibody. Levels of β actin were assessed as a loading control (B). The expression of CD21 and HLA-II was assessed prior to infection with EBV-GFP (C). TRIM22 N155, TRIM22 D155 and empty vector transduced HEK293 cells were infected with a VSV-GFP construct for 6h using serial dilutions of virus and analyzed by flow cytometry (D). Error bars represent standard deviation of the GFP+ cells. (**P<0.01, ***P<0.001 Two-way ANOVA with Bonferroni tests).

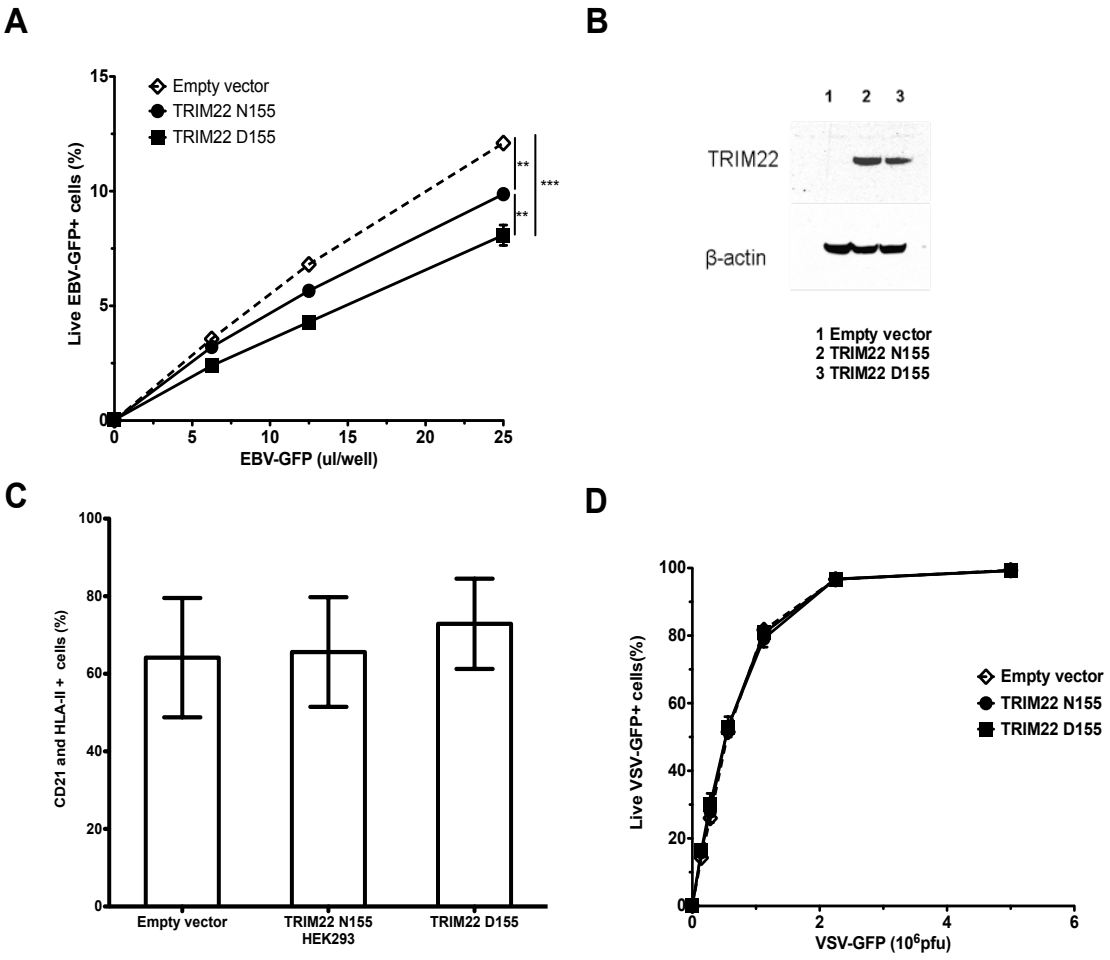

Figure 3.2 (Continued)

TRIM22 SNP variation correlates with EBV genome loads in B-LCLs

Because TRIM22 decreased the efficiency of EBV infection *in vitro,* and particular SNPs in *TRIM22* alter this efficiency, we next sought to determine the distribution of *TRIM22* SNPs in different ethnic populations in order to evaluate an indirect correlate of EBV infection, or a measurement of the EBV genome copy number in B-LCLs. The literature is unclear as to which B-LCL clone grows out in a group of EBV-transformed B cells [188], the number of virions required to transform a B cell, the basis of inheritance of the EBV copies and what controls the amplification of the EBV genome upon transformation. It is known that upon infection, linear EBV genomes circularize to form the episomes characteristic of latency approximately 20h post infection [189]. Although it is suggested that this one episome converts to a closed covalent circle around 36h post-infection, the copy number of EBV latency is restricted to 1-5 copies/cell. In addition, the large variation in copy number is also due to a 100-1000x amplification of the genome during latency.

Thus, we first determined whether there was a difference in the frequency of the *TRIM22* SNPs across different ethnic groups prior to assessment of EBV genome copy numbers in B-LCLs. To do so, we assessed previously available information available on B-LCLs from the HapMap consortium [180]. The HapMap B-LCLs originated from 4 different ethnic groups as described in the methods: Yoruba (YRI), Caucasians (CEU), Japanese (JPT) and Han-Chinese (HCB). These B-LCLs were previously genotyped by the HapMap consortium at two SNPs at the *TRIM22* locus, rs7935564 encoding an asparagine to aspartic

acid amino acid substitution at position 155 (N155D) in the coiled-coil domain, and rs1063303 encoding a threonine to arginine amino acid substitution at position 242 (T242R) in inker L2 domain of TRIM22 [180]. The HapMap database did not have information for the two SNPs rs61735273 (S244L) and rs73404240 (T294L), encoding amino acid substitutions in the linker L2 domain. The HapMap database was then analyzed to determine the frequencies of the nsSNPs encoding the N155D and T242R amino acid substitutions in *TRIM22* in the 4 ethnic groups.

We observed that the frequencies of individuals homozygous for D at amino acid position 155 in TRIM22 (D/D) or R at amino acid position 242 in TRIM22 (R/R) varied between the ethnic groups. The HCB and JPT cohorts both had higher frequencies of D155D and R242R homozygotes than the CEU and YRI groups. This difference between the ethnic groups was more profound when comparing homozygotes for TRIM22 D155D (HCB= 72.1%, JPT = 61.6%, CEU = 12.4%, YRI = 22.3%) than homozygotes for TRIM22 R242R (HCB=66.7%, JPT= 79.5%, CEU = 31.7%, YRI = 45.0%).

Because reports in the literature suggest there are potential cellular factors that influence EBV genome copy number in B-LCLs [190,191], we evaluated whether *TRIM22* SNPs could contribute to differences in EBV copy numbers in B-LCLs. A study conducted by Choy et al. (2008) measured EBV copy numbers in DNA extracted from HapMap B-LCLs by qPCR for the EBV DNA polymerase gene *BALF5* [192]. The average values of the EBV copy numbers were calculated for each ethnic group. The HCB and JPT ethnic groups

had mean EBV genome copy numbers of 0.28 and 0.15 copies per cell, respectively, whereas the CEU and YRI ethnic groups had higher mean EBV genome copy numbers of 2.11 and 0.69 copies per cell, respectively (Figure 3.3). Therefore, the genetic variation in *TRIM22* across ethnic groups correlates with the mean EBV genome copy numbers in B-LCLs from these ethnic groups. Specifically, the HCB and JPT groups that have higher frequencies of homozygotes encoding D155D and R242R also had lower EBV genome copy numbers in B-LCLs in comparison to the CEU and YRI ethnic groups.

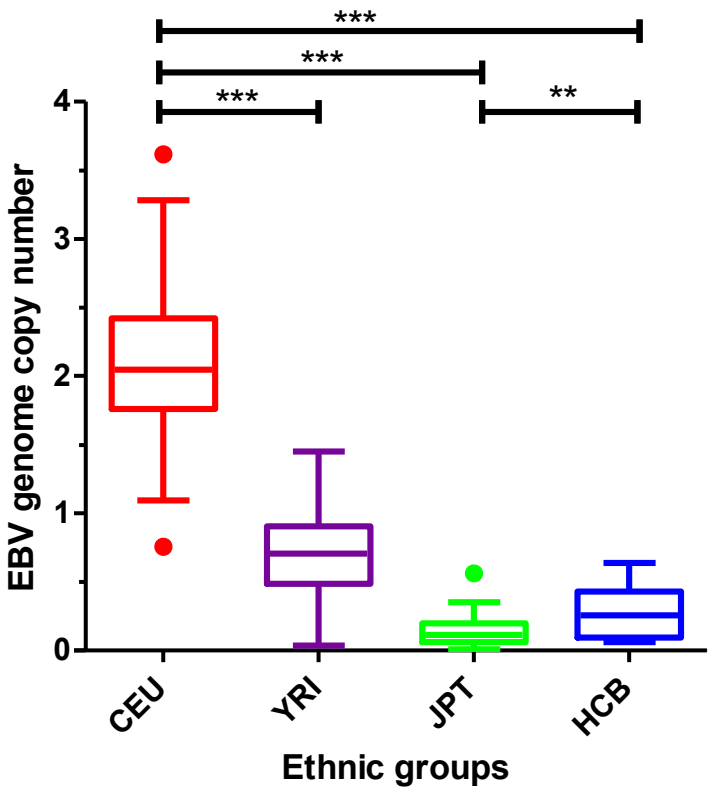

Figure 3.3: EBV genome copy number differs in B-LCLs from different cohorts. The EBV genome copy numbers as measured by qPCR for BALF5 by Choy E., et al., in B-LCLs from a cohort of 30 trios (90 individuals) from Yoruba in Ibadan, Nigeria (YRI); a cohort of 30 trios with Utah with Northern and Western European ancestry (generated at the Centre d'Etude Polymorphisme, CEU); from a cohort of 45 Japanese in Tokyo, Japan (JPT) and a cohort of 45 self-identified Han Chinese in Beijing, China (HCB) are shown.

We then compared the mean EBV genome copy numbers in B-LCLs from each ethnic group to the frequencies of homozygotes for D155D and R242R in each group (Figure 3.4A). We observed that the mean EBV genome copy numbers in B-LCLs correlated inversely with the frequency of individuals homozygous for TRIM22 D155D (Figure 3.4B) or TRIM22 R242R (Figure 3.4C) across the ethnic groups. We identified that a correlation between high TRIM22 SNP frequencies encoding the N155D and the T242R amino acid substitutions and low EBV genome copy numbers was consistent even when the EBV genome copy numbers in the LCLs were measured at baseline and when the LCLs were expanded, but not when measured in a smaller sample size of biological replicates (Data not shown).

At this point, it was unclear whether the lower EBV genome copy numbers were due to the ethnicity the B-LCLs originated from, or whether it was due to the *TRIM22* genotype differences in these B-LCLs. Hence, we assessed the correlation between *TRIM22* SNPs and EBV genome copy within the four ethnic groups, by subgrouping B-LCLs based on whether the *TRIM22* gene locus was homozygous or heterozygous at amino acid residues at position 155 (N/D) and position 242 (R/T). We observed that EBV genome copy numbers in the CEU cohort were approximately 2 copies per cell and were comparable between the different subgroups (Figure 3.4D). However, the EBV genome copy numbers were significantly different across *TRIM22* genotypes in the YRI cohort, despite the lack of a clear stratification (Kruskal-Wallis test, *P*-value 0.0459) (Figure 3.4E). This is possibly due to the significantly lower EBV genome copy numbers

in B-LCLs from TRIM22 D155D homozygotes versus the TRIM22 N155N homozygotes in the YRI cohort (Kruskal-Wallis test, P-value 0.0174) (Results not shown). Therefore, the inverse correlation of high frequencies of homozygotes encoding TRIM22 D155D or TRIM22 R242R with low mean EBV copy numbers was absent when the copy numbers were analyzed in relation to both these SNPs at the *TRIM22* gene locus. Therefore, these results argue that there are other factors besides *TRIM22* that affect EBV genome copy numbers in B-LCLs.

However, in the HCB and JPT cohorts, there is a potential premise for evolutionary pressure on *TRIM22,* as both cohorts only had R242R homozygotes, with no other genotypes present in the sampled B-LCLs (Figures 3.4F and 3.4G). Although this observation may be due to the smaller sample sizes of these ethnic groups than the CEU and YRI cohorts, it also hints at an evolutionary pressure that selects against the potentially more permissive TRIM22 T242T. Future studies should account for these sample sizes when investigating *TRIM22* genetic diversity.

These findings argued that the distribution of *TRIM22* SNPs in the coiled-coil and linker L2 domain differ between ethnic groups and initially suggested that these SNPs correlated with EBV genome copy numbers in B-LCLs from these groups. However, further analyses demonstrated that the ethnic origin of these B-LCLs is a better correlate for EBV genome copy numbers. This is consistent with the concept that multiple genetic traits are required to develop clear correlates for phenotypes, such as the maintenance of EBV genome copy numbers in B-LCLs.

This suggests that the *in vitro* culture conditions of the LCLs are variables that must be tightly controlled in future studies. This has also been demonstrated in a previous study that EBV DNA copy number fluctuates over time but stays in a constant range and does not correlate with culture conditions or cell density.

Figure 3.4: The frequency of TRIM22 SNPs correlates inversely with mean EBV genome copy number between different ethnic groups, but not within the ethnic groups. A schematic of TRIM22 with the amino acids of interest is shown (A). The mean EBV genome copy number in the CEU (red), YRI (purple), JPT (green) and HCB (blue) cohorts is shown relative to the frequency of D155D homozygotes (B) and R242R homozygotes (C) in these cohorts. The EBV genome copy numbers relative to the TRIM22 SNPs encoding either N/D at position 155 and either T/R at position 242 in the CEU (D), YRI (E), HCB (F), and JPT (G) ethnic groups is shown. *P* values were calculated using Kruskal-Wallis test with Dunn's multiple comparisons t test.

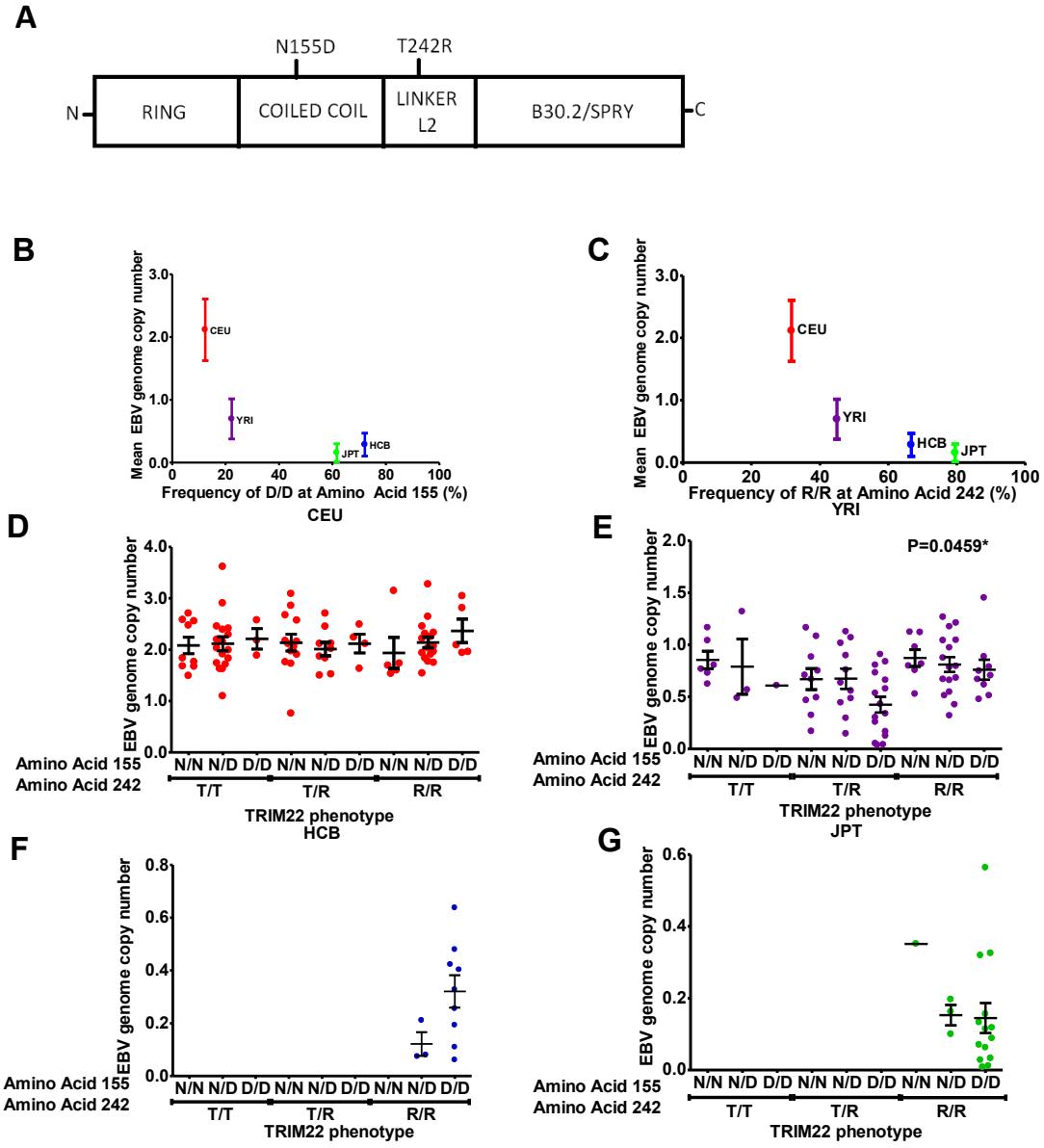

Figure 3.4 (Continued)

TRIM22 SNP correlates with variation in anti-EBV antibody titers.

Previous genome-wide association studies suggested that there is a correlation between specific genetic variation and anti-EBV antibody titers *in vitro* [193]. Upon measuring anti-EBV viral capsid antigen (VCA) IgG antibodies by ELISA in a cohort of patients from Malawi, we identified that patients homozygous for aspartic acid at position 155 (TRIM22 D155D) in the coiled-coil domain of TRIM22 had lower antibody titers compared to patients homozygous for asparagine at this position (TRIM22 N155N) (Figure 3.5). Although these results were consistent with the data observed in *in vitro* infections (Figure 3.2C), the interpretation was unclear. Prior literature suggests that seropositivity for anti VCA IgG titers alone is suggestive of a recent EBV infection or poorly functioning cellular immunity [194]. Further research that measures the anti-VCA IgM and anti-EBNA IgG titers must be conducted before drawing any conclusions from these titers. Therefore, these antibody titers are indicative of a past EBV infection, reactivation from latency, the immune status of these patients, or a combination of all or more of these factors.

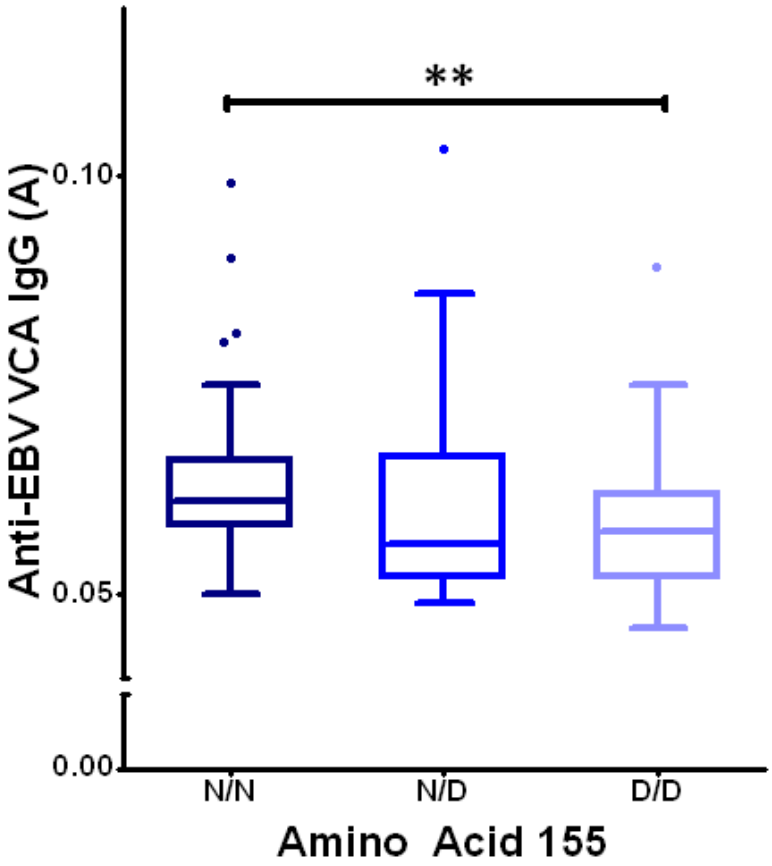

Figure 3.5: Individuals encoding TRIM22 D155D have lower anti-EBV antibody titers than individual encoding TRIM22 N155N. Plasma from a cohort of patients in Malawi was assessed for anti-EBV viral capsid antigen antibody titers by ELISA and optical densities were measured.

Discussion

Variation in TRIM22 linker L2 and coiled-coil domains alters host herpesviral susceptibility.

We also identified that variation in the linker L2 domain and the coiled-coil domain of TRIM22 altered the target cell's susceptibility to HSV-1 and EBV respectively. The linker L2 domain is arguably more important, as the TRIM22 haplotypes encoding an arginine at position 242 demonstrated lower HSV-1 viral yields, and the only genotype present in B-LCLs from the HCB and JPT cohorts was homozygozity for R242R.

Amino acid substitutions in the linker L2 domain have been shown to alter the function of TRIM proteins. This domain was demonstrated to be an important component for TRIM5 dimerization, in concert with the B-box 2 and coiled-coil domains [195]. Furthermore, an amino acid substitution from aspartic acid to glycine in the linker L2 domain of TRIM5 attenuated its anti retroviral activity against HIV-1 and HIV-2 in *in vitro* studies [195]. This amino acid substitution was also associated with higher HIV-1 susceptibility in Japanese and Indian populations [196]. In prior work on TRIM22, the arginine to threonine amino acid substitution at position 242 (R242T) has been associated with decreased anti-HIV-1 activity [181].

Interestingly, the R242T amino acid substitution in the linker L2 domain changed the distribution of TRIM22 from nuclear punctae to diffuse localization in the nucleus and cytoplasm in transfected HeLa cells [181]. This is consistent with our observation that *TRIM22* haplotypes 6 and 7, which encode R at position

242, with the adjoining leucine residue at position 244 and threonine residue at position 294, demonstrate greater restriction to HSV-1 infection *in vitro* relative to the other haplotypes or the empty vector. These results argue that it would be important to assess the subcellular localization of the different *TRIM22* haplotypes, particularly those encoding an arginine at position 242, when addressing their antiviral role in HSV-1 infection.

Furthermore, the absence of the *TRIM22* SNP encoding a threonine at position 242 in B-LCLs from the HCB and JPT populations suggests that these populations have a lower frequency of the more permissive *TRIM22* SNP. This argues for the selection of "restrictive" *TRIM22* haplotypes in these ethnic groups. However, increasing the power of these studies and conducting further validation studies will further inform this hypothesis. In summary, these findings all emphasize the importance of the amino acid substitutions in the linker L2 domain in the anti-viral activity of TRIM22.

There is a precedent for the importance of the coiled-coil domain in the anti-viral activity of the TRIM proteins. Amino acid residues in the coiled-coil domain of TRIM proteins, specifically in rhTRIM5α, have been shown to be under positive selection [197]. From studies on domain swaps in the rhTRIM5α protein, the coiled-coil region has been shown to be important in forming multimers, which is crucial for effective binding of rhTRIM5α to lentiviral capsids [57,198-200]. This domain has been also shown to be important in mediating HIV-1 capsid specificity [173,201]. However, one contrasting study suggests that the functional replacement of the coiled-coil domain of rhTRIM5α with the coiled-coil

domains of either the TRIM6 or TRIM34 paralogs maintains anti-HIV-1 activity [79].

We also provide further evidence for the functional importance of amino acid substitutions in the coiled-coil domain of TRIM22: (i) We identified that cells transduced with TRIM22 D155D show relatively lower efficiency of EBV-GFP infections *in vitro* relative to TRIM22 N155N, (ii) B-LCLs from ethnic groups with a higher frequency of individuals homozygous for TRIM22 D155D have lower EBV genome copy numbers on average and (iii) plasma from patients homozygous for D155D demonstrate lower anti-EBV VCA IgG antibody titers than patients homozygous for N155N. Although these are all indirect correlates of herpesviral infections and measure different parameters of the EBV replication cycle, we hypothesize that this amino acid substitution is important for TRIM22's anti-viral activity.

This data is inconsistent with the HSV-1 restriction observed in transfection systems with the *TRIM22* haplotypes that encode an aspartic acid at position 155 in the coiled-coil domain, or *TRIM22* haplotypes 2, 5 and 7. We reported that transfection of *TRIM22* haplotypes 5 and 7 both lowered HSV-1 yields relative to the empty vector. The *TRIM22* haplotype 2, on the other hand, did not demonstrate HSV-1 restriction. Both *TRIM22* haplotypes 5 and 7 encode an arginine at position 242 in the linker L2 domain, whereas *TRIM22* haplotype 2 does not. Therefore, two out of the three haplotypes that encode an aspartic acid at position 155 in the coiled-coil domain also encode the more potentially restrictive arginine at position 242 in the linker L2 domain. Both the haplotypes

that do so also demonstrate restriction against HSV-1. Therefore, it would be interesting to assess the importance of the arginine at position 242 in the linker L2 domain in TRIM22-mediated restriction activity against EBV. Further structural and functional studies assessing the individual and combined importance of these amino acids at these positions in the linker L2 and coiled-coil domains of TRIM22 are needed to interpret the anti-herpesviral role of TRIM22.

In contrast to previous *in silico* analysis of the *TRIM22* gene locus, which identified 9 nsSNPs in the B30.2/SPRY domain, it is noteworthy that the *TRIM22* variation was primarily in the linker L2 domain and the coiled-coil domain, in the traditional sequencing analysis of B-LCLs done by our collaborators. The only common nsSNP between these two studies is the S244L amino acid substitution in the linker L2 domain of TRIM22. Therefore, it is worth assessing the importance of this particular amino acid substitution in structural and functional studies of TRIM22.

Co-evolution of the TRIM5 and TRIM22 gene loci with herpesviruses

The Red Queen hypothesis proposed by Leigh Van Valen in 1973 argues that hosts must keep pace with competitors or risk facing extinction [202]. In the case of genetic variation, there is always an evolutionary advantage to be gained by innovation [203]. As such, the *TRIM5* and *TRIM22* gene neighbors evolved under positive selection in hominoids and Old World Monkey species, such that they were positively selected in different species [116]. The rhTRIM5α inhibits HIV-1 [152], whereas TRIM22 in humans shows significant HIV-1 inhibition

[94].Thus far, this discordant evolution in the *TRIM5* and *TRIM22* genes was attributed to coevolution of nonhuman primates with lentiviruses.

This pattern is similar to what is observed in the anti-herpesviral activity of the TRIM proteins. We previously found that the rhTRIM5α protein was capable of cross-species restriction of human HSV-1 [135], and we report here that the human TRIM22 protein also has an ability to restrict human HSV-1. In contrast, human TRIM5 does not restrict herpesviruses as much as the rhTRIM5alpha [135]. These results suggest that TRIM22 is a functional anti-herpesviral and anti-retroviral TRIM protein in humans, consistent with this protein being positively selected for in hominoids.

Further evidence for herpesviruses also exerting positive selection on TRIM proteins is evident in the *TRIM22* haplotypes that show discordant antiretroviral and anti-herpesviral activity. We demonstrate that the seven different haplotypes of *TRIM22* show varying degrees of HSV-1 inhibition. The nsSNP encoding the T242R amino acid substitution in the linker L2 domain of TRIM22 has previously been demonstrated to alter viral inhibition [93,163,164]. In the context of HIV-1, 293T cells transfected with *TRIM22* haplotypes homozygous for R242R show a decreased capacity to inhibit HIV-1 transcription [164]. In addition, HeLa cells transfected with the TRIM22 R242R also did not inhibit HIV-1 Gag production relative to wild-type TRIM22 [94]. In contrast with HSV-1, we observed that HeLa cells and CF2TH cells transfected with TRIM22 R242R inhibit HSV-1. These differences in the antiviral specificity exerted by these *TRIM22* haplotypes encoding R242R further hint at evolutionary pressures

exerted on this gene locus by retroviruses and herpesviruses. Consequently, we speculate whether the dichotomy of the antiviral specificity of the *TRIM5* and *TRIM22* gene loci is pathogen-driven and due to selection pressures from herpesviruses, in addition to the previously known lentiviruses.

There is a precedent for related genes that have different antiviral specificities despite being closely related. For example, the *IFIT1* and *IFIT2* haplotypes recognize different caps on viral mRNAs, suggesting that the presence of alleles in this gene locus allows for antiviral specificity against unrelated RNA and DNA viruses [204,205]. We hypothesize that the *TRIM22* haplotypes we identified are examples of the arms race between host and pathogens, in this case, the relatively unrelated herpesviruses and retroviruses. It would be interesting to assess whether the *TRIM22* haplotypes that restrict HSV-1 are different from those haplotypes that restrict HIV-1. However, the mechanism for these differences in antiviral specificity is hard to interpret given that the herpesviruses and retroviruses differ in their major pathogen-associated molecular patterns and replication cycles. Further studies assessing the common targets between these two viruses should be conducted.

Chapter 4:

CONCLUSIONS AND SIGNIFICANCE

4.1: Summary

This dissertation addressed the importance of human tripartite motif 22 (TRIM22) in herpesviral infections, using herpes simplex virus 1 (HSV-1) as a primary example of this virus family. We report a novel host and virus interaction, the importance of the subcellular localization of this interaction, and show that genetic variation at the *TRIM22* gene locus alters susceptibility to the herpesviruses.

We reported that TRIM22 is a novel restriction factor against HSV-1. We also demonstrate that it significantly inhibits the replication of a mutant virus lacking the immediate-early viral E3 ubiquitin ligase, infected cell polypeptide 0 (ICP0), crucial for HSV-1 replication. TRIM22 restricts ICP0-null virus replication by promoting heterochromatinization of viral genomes post nuclear-entry. This reduces viral gene transcription and expression, and consequently viral yields. It must be noted that the TRIM22-mediated restriction is overcome by the wild-type equivalent of HSV-1 in an ICP0-dependent, but a degradation-independent mechanism. This mechanism of viral escape of a host restriction factor is in contrast to the ICP0-mediated degradation of other known restriction factors, including TRIM19/PML, IFI16 and components of the DNA damage repair pathway. Hence, the wild-type equivalent of the virus has other ICP0-dependent mechanisms that overcome the restriction in a degradation-independent manner. Therefore, this work characterizes a novel interaction between the host intrinsic immune response and the virus.

The subcellular localization of the host surveillance system for herpesviruses has been a controversial topic recently. Host sensors of “non-self” DNA have been shown to be located in both the nucleus [30] and the cytoplasm [28], which was hard to corroborate with the nuclear location of herpesviral DNA recognition [206]. Our preliminary results suggest that the nuclear localization signal in the C-terminal B30.2/SPRY domain of TRIM22 is important for the inhibition of the nuclear-replicating HSV-1. This finding is yet another example of constitutively expressed host factors that target viral DNA upon nuclear entry in host cells. We also demonstrated that the TRIM22-mediated restriction is broader than previously known and show that it is capable of restricting other herpesviruses that replicate in the nucleus, including cytomegalovirus (CMV), a β-herpesvirus and Epstein-Barr virus (EBV), a γ-herpesvirus. However, it must be noted that TRIM22 has been shown to inhibit encephalomyocarditis virus (ECMV), a virus that replicates in the cytoplasm, suggesting that TRIM22 also has non-nuclear mechanisms of restriction. Overall, this work adds to the growing literature that addresses the importance of subcellular localization of anti-viral mechanisms implemented by the host.

The importance of the host *TRIM22* in host antiviral responses is also evident in the positive selection exerted on this gene [116], resulting in its haplotypic diversification. Our collaborators identified four non-synonymous single nucleotide polymorphisms (nsSNPs) encoding amino acid substitutions in the linker L2 and the coiled-coil domains of TRIM22. We report that the combination of an arginine at position 242, leucine at position 244 and threonine

at position 294 in the linker L2 domain are important for HSV-1 restriction. Preliminary results also demonstrate that the presence of an aspartic acid at position 155 in the coiled-coil domain associated with lowered correlates of EBV infection in *in vitro* and *ex vivo* studies. Therefore, this work suggests that the linker L2 and coiled-coil domains are a potential interface for interaction between herpesviruses and host TRIM22. Importantly, the mutations in the different *TRIM22* haplotypes is another example of the ongoing arms race between host and viruses, with the TRIM proteins as representatives of the host side and the herpesviruses as mediators of the virus side of the conflict.

4.2: Discussion

4.2.1: TRIM22 as a restriction factor

4.2.1.1: How does TRIM22 inhibit HSV-1?

The herpesviral genome consists of “naked” dsDNA that is not associated with proteins, as is traditionally seen with eukaryotic DNA. Although we demonstrated that TRIM22 promotes the compaction of viral DNA into nucleosomes, and modifies chromatin into inactive heterochromatin, the exact mechanism by which TRIM22 mediates these processes is unclear.

An additional mechanistic concern is in the interpretation of the magnitude of the rescue in ICP0-null virus yields upon TRIM22 depletion, relative to the magnitudes observed for other restriction factors in the literature. The overall rescue in ICP0-null virus yields upon TRIM22 depletion is up to a 1000-fold. In contrast, the rescue in ICP0-null virus yields upon depletion of known restriction factors such as TRIM19/PML or IFI16 is only approximately 10-100 fold. In addition, the defect at each stage in the viral replication cycle due to TRIM22 is only approximately a few-fold. We propose two possible hypotheses for this degree in TRIM22-mediated rescue in viral yields.

One hypothesis is that small effects at initial stages in viral replication, such as the observed reduction in histone occupancy on viral gene promoters, results in greater downstream defects on HSV-1 replication cycle. Another hypothesis is that TRIM22 restricts HSV-1 in an indirect pathway through one of the other well-known intrinsic resistance factors such as IFI16, TRIM19/PML in ND10 bodies, or a yet unknown factor. As mentioned earlier, further support for

this hypothesis is that the degree in rescue of the ICP0-null virus upon TRIM22 depletion is substantially higher than what is observed upon depletion of each of these individual restriction factors of HSV-1[43,122]. The potential mechanisms and experiments that will address the latter hypothesis are discussed below.

4.2.1.2: TRIM22 and IFI16

The interferon γ inducible protein 16 (IFI16) is an attractive candidate as an indirect mediator for TRIM22's restriction activity for the following reasons: (i) Both TRIM22 and IFI16 have similar anti-herpesviral specificity against both HSV-1 and CMV. Our lab previously demonstrated that IFI16 restricts HSV-1, and we reported here that TRIM22 restricts HSV-1. IFI16 was shown to restrict CMV at an early stage in viral replication by impairing viral DNA synthesis after nuclear entry [207]. Although the TRIM22-mediated mechanism of CMV restriction is currently unknown, we demonstrated that TRIM22 depletion increased HCMV viral yields; (ii) Both TRIM22 and IFI16 have a similar anti-viral mechanism in HSV-1 infection- they interfere with viral gene expression by promoting heterochromatinization of viral DNA [43]; (iii) Both TRIM22 and IFI16 localize to the nucleus, suggesting at a location-specific cooperation between these proteins; (iv) Although TRIM22 does not have a DNA-binding domain, IFI16 has demonstrated DNA-binding activity [208]. Based on the DNA-binding capacity of IFI16, we hypothesize that TRIM22 restricts HSV-1 through an IFI16-mediated mechanism.

Despite the earlier mentioned similarities between TRIM22 and IFI16, they differ in the mechanistic details of epigenetic silencing. In HSV-1 replication, incoming viral genomes initially are loaded with histones such as H3 to form nucleosomes. This is followed by post-translational modifications of the histone tails in nucleosomes to form inactive heterochromatin or active euchromatin. In the case of IFI16 depletion in primary fibroblasts, there are no observed changes in total histone H3, but there is an observed increase in euchromatin marks and decrease in heterochromatin marks on viral gene promoters [43]. In contrast, TRIM22 depletion reduces total histone H3 and reduces heterochromatin marks on viral DNA, with an insignificant trend in increased euchromatin marks on viral DNA. These results suggest a mechanistic quandary where the protein with the DNA-binding ability, or IFI16, does not alter initial histone H3 association on viral DNA. In contrast, TRIM22 alters histone H3 association despite the lack of a DNA-binding domain.

One possible explanation for these mechanistic differences between TRIM22 and IFI16 is that these restriction factors are temporally separated in their restriction. TRIM22 may initially act to compact viral DNA through a yet unknown DNA binding protein, followed by a synergy with the IFI16-mediated post-translational modification of histone tails to increase heterochromatinization and reduce euchromatinization of viral DNA. Furthermore, the effect of IFI16 on ICP0-null virus replication is more evident at low MOIs, whereas the TRIM22-mediated rescue in ICP0-null virus replication is more evident at high MOIs in primary fibroblasts [43]. These results further suggest that it is unlikely that IFI16

is upstream of TRIM22. Therefore, future studies should address the effect of combined depletion of these proteins on viral yields at low MOIs to assess whether they synergize in their restriction abilities as hypothesized.

4.2.1.3: TRIM22 and TRIM19/PML in ND10 bodies

Although it is well established that ND10 bodies are recruited to incoming viral DNA genomes in the nucleus, it is unclear how exactly these nuclear structures mediate restriction [155,209-214]. This restriction is demonstrable by depletion of ND10 components such as tripartite motif 19 (TRIM19)/ promyelocytic leukemia protein (PML), speckled protein 100 (Sp100), Bloom syndrome helicase (BLM), hDaxx and α-thalassemia mental retardation X-linked (ATRX), which resulted in an increase in plaque-forming efficiencies and a rescue in herpesviral yields of mutant viruses [40,42,122,215-218]. TRIM19/PML is particularly important, as it is critical for the assembly of ND10 bodies [63]. Therefore, we speculated whether TRIM22 mediates its restriction activity through TRIM19/PML.

The hypothesis that an interaction between TRIM22 and TRIM19/PML contributes to restriction of HSV-1 replication is attractive for the reasons that: (i) TRIM proteins are hypothesized to oligomerize through their coiled-coil domains [78,219] (ii) TRIM22 and TRIM19/PML are located in the nuclei of primary fibroblasts (Figure 2.4, [220]). TRIM22 has been shown to localize to nuclear Cajal bodies in certain cell types [221], and TRIM19/PML is critical for nuclear ND10 body formation [63]. Lastly, ND10 bodies are commonly associated with

Cajal bodies [222]. (iii) TRIM19/PML has been shown to mediate the anti-viral activity of exogenous interferon (IFN) in ICP0-null virus infections at low MOIs [223]. This is similar to our reported results at low MOIs- TRIM22 mediates a few-fold rescue in viral yields of ICP0-null virus in Type I and Type II IFN pre-treated cells (iv) Both TRIM22 and TRIM19/PML have similar anti-herpesviral specificity. ND10 bodies, containing TRIM19/PML, have been implicated in the restriction of HSV-1, HCMV and EBV [214,224,225]. This is consistent with our results of TRIM22-mediated restriction of all three viruses. (v) TRIM19/PML depletion increases the viral yields of an ICP0-null mutant HSV-1, but not wild-type HSV-1 [218]. This is similar to the phenotype observed upon TRIM22 depletion in ICP0-null versus ICP0-rescued virus infections.

A potential mechanism as to how TRIM22 mediates HSV-1 restriction through TRIM19/PML is that TRIM22 cooperates with TRIM19/PML to form higher-order multimers with TRIM19/PML. Hypothetically, this would stabilize TRIM19/PML. Given that TRIM19/PML is crucial for the assembly of the ND10 bodies that localize to incoming viral genomes, we hypothesize that TRIM22 depletion would result in the destabilization of ND10 bodies, preventing ND10 function.

A preliminary test of the potential relationship between TRIM22 and TRIM19/PML is to assess whether these proteins colocalize at endogenous levels, and also in the context of viral infections. However, the literature has conflicting results on whether TRIM22 localizes to TRIM19/PML in ND10 bodies, with no clear results showing colocalization of TRIM22 and TRIM19/PML in

immunofluorescence studies. One report claims in “data not shown” that there is no observed colocalization between TRIM22 and TRIM19/PML in ABC28 cells, a breast cancer cell line, in both basal and progesterone-treated cells [221]. Another dissertation claims that over-expressed TRIM22 colocalizes with PML in HT1080 cells, a fibrosarcoma cell line [226]. The endogenous localization of TRIM22 is also unclear- the localization of TRIM22 has been attributed to centrosomes [117] and to nuclear bodies that colocalize with Cajal bodies constituting of p80-coilin [221]. TRIM22 also has been shown to change subcellular localization depending on the cell cycle phase [221]. In our immunofluorescence studies, we have been limited in our testing of TRIM22 subcellular localization by the lack of an effective antibody specific for endogenous TRIM22. To determine if there is any interaction between TRIM22 and TRIM19/PML, it will be important to assess whether a tagged TRIM22 construct and TRIM19/PML colocalize in immunofluorescence studies under conditions of ICP0-null virus and ICP0-rescued virus infections. Further co-immunoprecipitation experiments should also assess for a direct interaction between TRIM22 and TRIM19/PML.

A second means of interrogating the TRIM22 and TRIM19/PML relationship is by the measurement of ICP0-null HSV-1 viral yields upon depletion of these proteins. In preliminary studies, we depleted either TRIM22 or TRIM19/PML or both proteins, and assessed ICP0-null and –rescued viral yields in low MOI infections. We observed an additive effect on the rescue in ICP0-null virus yields upon depletion of both TRIM22 and PML, relative to either TRIM22 or

PML alone, in primary fibroblasts (Results not shown). These preliminary results suggest that TRIM22 and PML act in independent pathways in restricting the replication of the ICP0-null virus. These preliminary results also suggest that TRIM22 is unlikely to mediate HSV-1 restriction by PML; however, further experiments are needed to validate these results. In addition, other components of ND10 bodies, particularly hDaxx and ATRX, which have been shown to form a chromatin-remodeling complex in CMV restriction, should also be investigated [227].

4.2.1.4: TRIM22 and TRIM28/KAP1

4.2.1.4: TRIM22 and an unknown restriction factor

Alternatively, TRIM22 may mediate its restriction activity through any of the other known or unknown factors that have anti-herpesviral restriction activity. A formal experiment to test this would be to pull down TRIM22 with antibodies specific to TRIM22 or a tagged construct, and conduct mass spectrometry on interacting partners in the context of mock-, ICP0-null and ICP0-rescued virus infections. The interacting proteins of interest likely to mediate anti-viral activity would be those that are mutually present or absent in ICP0-null virus infections relative to the other conditions. Given that TRIM22 alters histone occupancy and histone modifications on the viral genomes, it would be interesting to determine whether any of the pulled down proteins include chromatin-remodeling enzymes, which are important in the context of HSV-1 gene expression [48,130,228-230].

Another approach to assess how TRIM22 mediates its restriction is by addressing the relative importance of the different domains, or the RING, B-box, coiled-coil, the connecting linker L2, and B30.2/SPRY domains. We and others demonstrated that the presence of the B30.2/SPRY domain is important for the nuclear localization of TRIM22. Besides conferring a nuclear localization for TRIM22 to the same compartment as the nuclear-replicating herpesviruses, there may be other factors that argue for the importance of this domain [142]. In the case of rhTRIM5α, the B30.2/SPRY domain has been shown to be important for the anti-viral capsid specificity [77,173,174,231]. However, given the nuclear localization of TRIM22 in our system, it is unlikely that TRIM22 is mediating its anti-herpesviral activity by binding herpesviral capsid, which gets docked at nuclear pores during viral replication. The B30.2/SPRY domain in TRIM22 has predominantly shown to be important in mediating TRIM22's role in NF-κB activation, suggesting that its role in innate immune activation may have a mechanistic role in HSV-1 inhibition.

We also demonstrate that the linker L2 and coiled-coil domains are important for anti-herpesviral activity - an arginine at position 242, leucine at position 244 and threonine at position 294 in the linker L2 domain is important for HSV-1 restriction. We also show that the amino acid substitution from an asparagine to an aspartic acid residue at position 155 in the coiled-coil domain of TRIM22 impacts EBV restriction. There is precedent for the importance of the linker L2 and coiled-coil domains in TRIM-mediated restriction. In fact, mutations in the linker L2 domain with the adjoining coiled-coil domain of TRIM5α promote

the formation of α-helices in the protein. These α-helices facilitate higher-order multimerization, and the assembly of cytoplasmic TRIM5α structures crucial for HIV-1 restriction [78,232]. In our system, we observe that exogenously expressed TRIM22 forms nuclear bodies. Thus, we propose that these amino acid substitutions in the linker L2 domain and the coiled coil domain of TRIM22 potentially increase the α-helical content in this protein. Subsequently, these secondary structures could promote higher order multimerization and the formation of nuclear bodies, important for HSV-1 restriction. Therefore, future immunofluorescence studies could address the subcellular localization of the different *TRIM22* haplotypes encoding these amino acid substitutions. Additional circular dichroism studies assessing the impact of these amino acid substitutions on TRIM22 secondary structures could also be conducted.

Notably, the TRIM22-mediated effect on HSV-1 restriction is not entirely abrogated by a RING domain deletion, or by the inactive E3 ubiquitin ligase mutant. This is in contrast to the previously known anti-viral activity of TRIM22, which in many cases is dependent on the E3 ubiquitin ligase activity of the RING domain [89-91,109]. Therefore, additional testing of whether the TRIM22 mediated restriction of HSV-1 is abrogated under conditions of treatment with a proteasomal inhibitor or MG132 would provide further evidence on the importance of the RING domain and consequently, the E3 ubiquitin ligase pathway in this restriction.

In order to identify novel TRIM22 interacting partners, future studies should assess the restrictive abilities of coiled-coil and linker L2 domain-deletion

mutants, and of mutants with specific amino acid substitutions demonstrated to be crucial for restriction. This would be followed up by pull-down studies comparing full-length TRIM22 versus deletion mutants lacking the domains or amino acid substitutions important for HSV-1 restriction to identify novel interactors.

4.2.1.5: How does HSV-1 overcome the TRIM22-mediated restriction?

It is interesting to note that the TRIM22-mediated restriction is only evident on the ICP0-null virus infections in primary fibroblasts, and this restriction is overcome by the wild-type equivalent of the virus. These results are suggestive of an ICP0-dependent mechanism of abrogating TRIM22 restriction. However, we demonstrated that TRIM22 is not degraded by ICP0.

ICP0 has been shown to overcome host immune response by the ICP0-mediated degradation of TRIM19/PML, which results in the disruption of ND10 bodies [218]. We hypothesize that ICP0 overcomes TRIM22-mediated restriction in a similar mechanism by the disruption of TRIM22 nuclear bodies. One potential mechanism is that ICP0 has been shown to localize to centromeres and degrade components important for the architecture of centromeres such as the centromeric proteins (CENPs), specifically CENP-A, CENP-B and CENP-C [233-236]. This results in an accumulation of p80-coilin on these damaged centromeres [237,238]. TRIM22 nuclear bodies colocalize with Cajal bodies containing p80-coilin [221]. TRIM22 and p80-coilin in Cajal bodies have been demonstrated to interact in co-immunoprecipitation studies [221]. Therefore, we

hypothesize that the presence of ICP0 destabilizes Cajal bodies, and subsequently TRIM22 nuclear bodies by promoting p80-coilin relocalization to damaged centromeres. This potentially disrupts TRIM22 nuclear bodies in their anti-viral functional role. It would be interesting to assess whether ICP0 disrupts TRIM22 bodies upon viral infection by immunofluorescence studies in primary fibroblasts.

A potential unbiased approach to assess how ICP0 overcomes the TRIM22-mediated restriction of HSV-1 originates from the cell-type specific differences we observed. The effect of endogenous TRIM22 in primary fibroblasts seems specific to the ICP0-null virus, and the ICP0-rescued viruses are capable of overcoming the restriction in high MOI infections. However, in HeLa cells, we observed that both the ICP0-null and –rescued viruses are capable of overcoming the exogenous TRIM22-mediated effect at high MOIs, despite the high levels of protein expression observed upon transfection (Results not shown). Instead, the TRIM22-mediated restriction is evident on both the ICP0-null and ICP0-rescued viruses at low MOIs in HeLa cells. These cell-type differences suggest there is a factor present in primary fibroblasts, but absent in HeLa cells, which is crucial for the ICP0-rescued virus's ability to overcome the TRIM22 restriction. A formal experiment assessing TRIM22's interacting proteins in ICP0-null virus versus ICP0-rescued virus infections in primary fibroblasts versus HeLa cells could be informative of how ICP0 overcomes the TRIM22-mediated restriction in these cell types.

Overall, it must be noted that the fact that ICP0 overcomes the TRIM22-mediated restriction of HSV-1 in primary cells is further evidence that the herpesviruses have co-evolved with their hosts, and is an additional example for the virus-host arms race. Future studies could assess whether there are viral proteins parallel to ICP0 in the β- and γ-herpesviruses that can overcome the TRIM22-mediated restriction. Potential candidates include the HCMV viral proteins, IE1 and pp71, which have been shown to complement the ICP0-null HSV-1 [239]. In the case of EBV, the immediate-early protein, BZLF1, has been shown to disrupt ND10 bodies much like one of the functions of ICP0 [225]. We observed that TRIM22 depletion increased HCMV viral yields by approximately 4-fold whereas TRIM22 over-expression reduced the efficiency of EBV infection *in vitro* by approximately 3-fold. It must be noted that the viruses used in both experiments are equivalent to the wild type, and therefore we hypothesize that replication defective versions of these viruses, such as an IE1-null and pp71-null HCMV mutant virus or a BZLF1-null EBV mutant virus, are likely to be restricted to a greater extent.

4.2.2: TRIM proteins as epigenetic regulators

Epigenetic silencing is mediated by factors that either add, remove, read and transmit repressive and active histone modifications, DNA methylation and acetylation marks on chromatin. This dissertation reports a novel function of TRIM22 as an epigenetic regulator of viral genes. This was evidenced in conditions of TRIM22 depletion in primary fibroblasts by reduced nucleosomal

loading, reduced heterochromatin histone modifications and a trend towards an increase in euchromatin histone modifications immunoprecipitated on immediate-early viral gene promoters.

The premise for TRIM proteins as modifiers of epigenetic processes exists in a subset of the TRIM superfamily of proteins that have a C-terminal PHD-Bromodomain chromatin interacting module [240]. These TRIM proteins differ from TRIM22 in their C-terminal domain, where TRIM22 has a B30.2/SPRY domain. Examples of such TRIM proteins include TRIM24 (TIF1α), TRIM28 (TIF1β) and TRIM33 (TIF1γ). In the context of viruses, TRIM28 corepressor complex was shown to mediate retroviral silencing in embryonic stem cells. TRIM28 binds the retrovirus primer binding site adjacent to the 5' long terminal repeat and mediates the addition of a dimethyl group on H3K9 [241]. TRIM28 starts this process by the recruitment of epigenetic silencing factors such as histone deacetylase, histone methyltransferase, and members of the heterochromatin protein (HP1) family [242]. The authors also hypothesize that TRIM28 is targeted to DNA by a Kruppel-associated box zinc finger (KRAB-ZNF) protein. Therefore, TRIM28 acts as a corepressor by tethering epigenetic effectors to the target DNA site on the retroviral primer binding site, resulting in condensed nucleosomes that repress transcription and viral gene expression.

Although members of this protein family are implicated in viral processes, their role in epigenetic regulation of the viral replication cycle is unclear. For example, the adenovirus E4orf3 viral gene product targets TRIM33 for proteasome-dependent degradation in infection. In addition, both TRIM24 and

TRIM33 are reorganized to TRIM19/PML bodies in viral infection suggesting that the regulation of these proteins is important for adenoviral replication [243]. It has been suggested that the E4-ORF3 viral oncoprotein mediated reorganization inactivates p53, TRIM19/PML, TRIM24. MRE11/RAD50/ NBS1 (MRN) tumor suppressor complex, enabling small DNA tumor virus mediated dysregulation of the cell cycle [244]. Additionally, TRIM24 and TRIM33 were shown to form a co-repressor complex and cooperatively repress the VL-30 class of endogenous retroviruses in the liver [243]. TRIM24 is also shown to be downregulated in the increased pathogenicity of a virulent mouse-adapted strain of influenza [245]. Additionally, TRIM33 was shown to be upregulated in duck tracheas infected with a highly pathogenic or a low pathogenic strains of H5N1 avian influenza A viruses [189]. To summarize, there is precedence for the epigenetic role of TRIM proteins in viral processes and we add TRIM22 to this list.

4.2.3: TRIM22 as an immune effector

4.2.3.1: TRIM22 in the adaptive immune system

This dissertation does not address the importance of TRIM22 as an immune effector, despite the significant amount of literature suggesting its importance as an immune gene [246]. The role of TRIM22 in the adaptive immune system is closely tied to its expression, as evidenced by: (i) its high expression in cells of the lymphoid lineage [247] (ii) its decreased expression upon T cell activation [247] and repression during T cell costimulation [99] (iii) its association with autoimmune disorders, including the observed higher expression

of TRIM22 in lesional psoriatic skin relative to non-lesional psoriatic skin in patients [248] (iv) In systemic lupus erythematous (SLE), there was an observed hypomethylation of the *TRIM22* gene locus in naïve $CD4^+$ T cells from patients [249], and TRIM22 expression was decreased in $CD4^+$ T cells in a "disease active" state relative to $CD4^+$ T lymphocytes in a "nonactive" state from a SLE patient [100].

It must be noted that it was unclear in these studies whether the role of TRIM22 is causative or phenotypic. In the psoriasis study, the authors hypothesized that the higher expression of TRIM22 and other anti-viral genes resulted in the infrequent cutaneous viral infections observed in psoriatic patients [248]. In the SLE study, the authors assumed that the observed downregulation of TRIM22 is a marker of CD4+ T cell activation, consistent with the presence of activated lymphocytes in an autoimmune lymphocyte phenotype [100]. Interestingly, the $CD4^+$ T cells in the SLE "disease active" state also demonstrated a higher expression of DNA virus associated proteins, including heterogeneous nuclear ribonucleoprotein U-like protein 1 (HNRPUL1), which binds an adenovirus oncoprotein and tumor necrosis factor ligand superfamily member 14 (TNFSF14), which is the herpes virus entry mediator ligand [100]. Although unreported, it would be interesting to assess if the higher expression of these DNA virus associated proteins correlates with higher susceptibility to viral infections in these patients.

In conclusion, these findings argue that it is worth addressing TRIM22's role in *in vivo* implications for herpesviral treatments. Although the anti-viral role

of TRIM22 suggests that increasing its expression would be advantageous to the host for viral inhibition, the counter argument would be that TRIM22 downregulation in lymphocytes might be required for T cell activation in order to mount an effective immune response. Despite the lack of clarity on the *in vivo* role of TRIM22 in the adaptive immune system, TRIM22 may well be one of many factors in the balance between the “good” or anti-viral immune responses versus the “bad” or autoimmune disorders.

4.2.3.2: TRIM22 in the innate immune system

TRIM22 has been shown to have a role in innate immune regulation of nuclear factor kappa B (NF-κB) activation, a transcription factor important in the induction of inflammatory cytokines [101,102,119]. The reports on TRIM22’s role in NF-κB activity suggest that it is a balancing factor between the canonical and non-canonical pathways of NF-κB activation. We wished to address whether TRIM22’s role in the innate immune system is separable from its role as a restriction factor in herpesviral inhibition.

The canonical pathway of NF-κB activation is regulated by the tumor necrosis factor receptor associated factor 6 (TRAF6), an E3 ubiquitin ligase (Reviewed in [102]). TRAF6-mediated ubiquitination activates the downstream kinase complex composed of TAK1, TAB1 and TAB2. This kinase complex phosphorylates the inhibitor of κB (IκB) kinase (IKK) complex. This is mediated by TAB2, which binds the polyubiquitin chains of TRAF6, which in turn promotes TRAF6 self-ubiquitination and its association with the downstream IKK complex.

This results in the subsequent dissociation of IκBα from NF-κB, and thus NF-κB activation. TRIM22 has been shown to regulate this pathway by targeting TAB2 for degradation, and thus it is a negative regulator of the canonical pathway of NF-κB activation [102].

Another group addressed the role of TRIM22 as a positive regulator in the non-canonical pathway of NF-κB activation. These studies are conducted by over-expression of TRIM22, rather than by co-transfection studies of TRIM22 and TRAF6 as reported earlier. In the non-canonical pathway, TRIM22 phosphorylates IKKα, resulting in the subsequent activation of the NF-κB pathway [101]. These results are consistent with the initial report that TRIM22 overexpression resulted in NF-κB activation in a macrophage cell line [119].

We hypothesize that HSV-1 hijacks TRIM22's role in regulating the canonical versus non-canonical pathways of NF-κB regulation. It has been shown that ICP0 expression exports herpesvirus-associated ubiquitin-specific protease (HAUSP) or ubiquitin-specific protease 7 (USP7), from the nucleus to cytoplasm where USP7 binds and deubiquitinates TRAF6 and IKKγ, thus inhibiting the NF-κB response. Hence, the presence of ICP0 in a wild-type virus infection would be reminiscent of TRIM22's role in the canonical pathway or where TRIM22 is an inhibitor of the NF-κB response. Conversely, in the absence of ICP0, TRIM22 potentially mediates NF-κB activation via the non-canonical pathway resulting in the production of NF-κB responsive anti-viral cytokines. In fact, TRIM22 has been hypothesized to interact with USP7 in a yeast two-hybrid screen under non-viral conditions [250]. It would be interesting to assess if

TRIM22 sequesters USP7 in the nucleus, preventing the USP7-mediated inhibition of the NF-κB response observed in the presence of ICP0.

Consistent with this hypothesis, we observed that TRIM22 depletion reduced basal levels of NF-κB cytokines, such as IL6 transcript levels in untreated primary fibroblasts (Results not shown). We also observed that TRIM22 depletion reduced the induction of IL-1beta and IL-18, other NF-κB responsive cytokines by a few fold in the ICP0+ or 7134R virus infection (Results not shown). There was no difference observed upon ICP0- or the 7134 virus infection (Results not shown). However, the more conclusive experiment is to determine whether the rescue in ICP0-null viral yields observed under TRIM22 depletion is abrogated upon activating the NF-κB pathway. Therefore, the role of TRIM22 in regulating the NF-κB pathway in HSV-1 infection would require further elucidation.

Interestingly, there may be a role for TRIM22 as an innate immune effector in RNA virus infections. In preliminary results, we observed an eight-fold reduction in IFNβ induction upon TRIM22 depletion in primary fibroblasts relative to control depletion in Sendai virus infection, an RNA virus (Results not shown). These preliminary results suggest that the TRIM22-mediated effect in the inhibition of RNA viruses may be dependent on its role as an innate immune effector and is potentially separable from its mechanism of herpesvirus inhibition.

4.2.4: TRIM22 as a cell cycle mediator in oncogenesis

There is evidence in the literature that suggests that TRIM22 is involved in the progression of the cell cycle [251]: (i) TRIM22 is downregulated in breast cancer [252] (ii) its overexpression reduces the clonogenic growth of U937 cells, a leukemic cancer cell line [118] (iii) it is downregulated upon maturation of hematopoietic stem cells into the erythroid lineage [103] (iv) it associates with the centrosome regardless of the cell cycle phase in one report [117], but TRIM22 nuclear bodies begin to form in the G_0/G_1 phase of the cell cycle and are dispersed in the S phase in HeLa cells [221] (iv) its lower expression in Wilms tumors, a renal neoplasm, has also been associated with fatality [104,105] and its reduced expression has been associated with neuroblastoma tumorigenesis [253].

Although it is well-known that the small DNA tumor viruses such as SV40 and the human papilloma virus require an S-phase environment to support viral replication, herpesviruses prevent cell cycle progression into the S phase during lytic replication. HSV-1 has been shown to block cellular DNA synthesis [254], HSV-1 ICP0 arrests cell cycle progression from G_1 into the S phase [255-257] and ICP27 is also important in HSV-1 mediated growth arrest [258]. CMV has been shown to inhibit cell cycle progression from G_1 to S phase, despite encoding proteins that elicit cell cycle progression such as IE1 and IE2 (reviewed in [258]). In EBV infection, the switch from latent replication to lytic replication results in a G_0/G_1 arrest (reviewed in [258]). Therefore, there is precedent for the importance of cell cycle arrest in herpesviral replication. We speculate that the

interaction between TRIM22, the herpesviruses and the cell cycle could begin to be elucidated by assessing viral yield assays in synchronized cells, and in growth-arrested cells.

As we mentioned previously, TRIM22 potentially has a role in viral latency. The EBV latent membrane protein-1 (LMP-1), which is required for EBV latency, is upregulated in exogenous TRIM22 expression [112]. TRIM22 also has been demonstrated to be upregulated by the KSHV LANA protein [111]. Although there have been no direct experiments addressing the importance of TRIM22 in latency, it would be interesting to address whether the γ-herpesviruses such as EBV hijack the cell biological role of TRIM22 in cell proliferation in mediating oncogenesis post establishment of viral latency.

4.2.5: Is *TRIM22* genotype a predictor of host herpesviral susceptibility?

The *TRIM5* and *TRIM22* gene neighbors have been shown to be important in restricting lentiviruses [259,260]. Additional data suggests that TRIM proteins are important for restricting viruses outside of the lentivirus family, particularly TRIM22 (Reviewed in [153]). We demonstrated in this dissertation that TRIM22 inhibits the herpesviruses and also showed that the seven different *TRIM22* haplotypes identified by our collaborators vary in their degree of HSV-1 restriction. We also show that individuals homozygous for the presence of SNPs encoding an arginine at position 242 in the linker L2 domain, or homozygous for an aspartic acid at position 155 in the coiled-coil domain are potentially more restrictive to HSV-1 and EBV, respectively. We demonstrate that the frequency of

individuals homozygous for the more “restrictive” SNPs varied across Caucasian, Yoruba, Japanese and Han Chinese ethnic groups. It would be interesting to assess whether the *TRIM22* haplotypes that a person encodes correlate with individual susceptibility to herpesviral infection/disease. Further studies assessing the *TRIM22* genotypes of individuals and their herpesviral titers at early time points post acquisition and measurement of herpesviral loads post reactivation as a correlate of herpesviral disease may be potential avenues of interest.

The haplotypic diversification observed at the *TRIM22* gene locus, combined with additional studies addressing the role of the different *TRIM22* haplotypes in altering the degree of susceptibility to other relevant viruses in the human population including human immunodeficiency virus-1 (HIV-1), hepatitis B virus (HBV), hepatitis C virus (HCV), encephalomyocarditis virus (EMCV) and influenza A virus (IAV) will further broaden the spectrum of evidence for the importance of TRIM22 as an anti-viral factor.

In conclusion, we demonstrated that TRIM22 is a novel restriction factor of herpesviruses with a focused analysis on the mechanism of TRIM22-mediated restriction in HSV-1 replication. The genetic variation in *TRIM22* is a potential marker for the differences in herpesviral susceptibility between individuals and across ethnic groups, and is another example of the arms race between hosts and viruses.

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